

**Development of a Bacteriophage-based Biopesticide for Fire Blight**

by

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## **Abstract**

Fire blight is an economically important disease of apples and pears that is caused by the bacterium *Erwinia amylovora*. Control of the disease depends on limiting primary blossom infection in the spring, and rapidly removing infected tissue. The possibility of using phages to control *E. amylovora* populations has been suggested, but previous studies have failed to show high treatment efficacies. This work describes the development of a phage-based biopesticide that controls *E. amylovora* populations under field conditions, and significantly reduces the incidence of fire blight.

This work reports the first use of *Pantoea agglomerans*, a non-pathogenic relative of *E. amylovora*, as a carrier for *E. amylovora* phages. Its role is to support a replicating population of these phages on blossom surfaces during the period when the flowers are most susceptible to infection. Seven phages and one carrier isolate were selected for field trials from existing collections of 56 *E. amylovora* phages and 249 epiphytic orchard bacteria. Selection of the phages and carrier was based on characteristics relevant to the production and field performance of a biopesticide: host range, genetic diversity, growth under the conditions of large-scale production, and the ability to prevent *E. amylovora* from infecting pear blossoms. *In planta* assays showed that both the phages and the carrier make significant contributions to reducing the development of fire blight symptoms in pear blossoms.

Field-scale phage production and purification methods were developed based on the growth characteristics of the phages and bacteria in liquid culture, and on the survival of phages in various liquid media.

Six of twelve phage-carrier biopesticide treatments caused statistically significant

reductions in disease incidence during orchard trials. Multiplex real-time PCR was used to simultaneously monitor the phage, carrier, and pathogen populations over the course of selected treatments. In all cases the observed population dynamics of the biocontrol agents and the pathogen were consistent with the success or failure of each treatment to control disease incidence. In treatments exhibiting a significantly reduced incidence of fire blight, the average blossom population of *E. amylovora* had been reduced to pre-experiment epiphytic levels. In successful treatments the phages grew on the *P. agglomerans* carrier for 2 to 3 d after treatment application. The phages then grew preferentially on the pathogen, once it was introduced into this blossom ecosystem. The efficacy of the successful phage-based treatments was statistically similar to that of streptomycin, which is the most effective bactericide currently available for fire blight prevention.

The *in planta* behaviour of *E. amylovora* was compared to that of *Erwinia pyrifoliae*, a closely related species that causes fire blight-like symptoms on pears in southeast Asia. Duplex real-time PCR was used to monitor the population dynamics of both species on single blossoms. *E. amylovora* exhibited a greater competitive fitness on Bartlett pear blossoms than *E. pyrifoliae*.

The genome of *Erwinia* phage  $\phi$ Ea21-4 was sequenced and annotated. Most of the 84.7 kB genome is substantially different from previously described sequences, though some regions are notably similar to *Salmonella* phage Felix 01 . Putative functions were assigned to approximately 30% of the predicted open reading frames based on amino acid sequence comparisons and N-terminal sequencing of structural proteins.

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### Abbreviations

CFU - colony forming units

EPS - exopolysaccharide

HR - hypersensitive response

MOI - multiplicity of infection; the ratio of phages to host cells (eg. when the concentration of phages is ten times that of host cells,  $MOI = 10$ )

$OD_{600}$  - optical density to light with a wavelength of 600 nm

ORF - open reading frame

PB - sodium phosphate buffer

$PB_{salt}$  - sodium phosphate buffer amended with 100 mM NaCl and 2 mM  $MgCl_2$

PCR - polymerase chain reaction

PFU - plaque forming units

RFLP - restriction fragment length polymorphism

TEM - transmission electron microscopy

## General Introduction

This work describes the development of a phage-based biopesticide for fire blight. Fire blight is a necrotic disease of rosaceous plants that is caused by the bacterial species, *Erwinia amylovora*. The pathogen is present in most apple and pear growing regions of the world, and causes serious economic losses to commercial growers in most of them (Bonn & van der Zwet, 2000). The disease cycle begins anew each spring when warm weather favours the growth and dispersal of *E. amylovora*. The pathogen colonizes open blossoms, multiplies, and infects the tree through natural openings in the blossoms (Wilson, Epton & Sigeo, 1989). Most control strategies are therefore focused on suppressing the growth of *E. amylovora* so as to prevent blossom infection. Currently, the most effective of these treatments is the timed application of streptomycin to open blossoms. However, specific concerns about streptomycin-resistant *E. amylovora*, along with the general trend to avoid antibiotic use in agriculture, are driving the development of alternative control strategies. In Canada, the registration of streptomycin for fire blight prevention is under constant scrutiny, and the registration of bacterial biopesticides has begun.

Bacteriophages, or simply “phages”, are viruses that infect bacterial cells. They consist of a nucleic acid genome contained within a protein or lipoprotein capsid. Upon infection, the phage-encoded gene products re-direct the host cell metabolism to cease normal function and instead devote its resources to replicating the phage. Once mature phage virions have accumulated, the bacterial cell is lysed, releasing a new generation of dozens or hundreds of infectious phages from a single initial infection.

The use of bacteriophages to control bacterial pathogens is an idea that dates back to the discovery of phages in the early 20<sup>th</sup> century. That discovery is formally attributed to independent investigations by Frederick Twort in 1915 and Felix d’Herrelle in 1917. D’Herrelle was the first to advocate the potential therapeutic uses of phages, perhaps because he first encountered them while working on another biological control problem (Summers, 1999). D’Herrelle’s first published report on phages described a filterable agent that killed the dysentery bacillus that is now known as *Shigella* (D’Herrelle, 1917). He noted that the agent was abundant in the stools of recovering patients, but rare or absent in those of acute patients, and suggested that the agent was an exogenous means of immunity. He concluded that the agent present in recovering dysentery patients was a particulate microbe infecting bacteria. Drawing on the Greek word *phagein* (eater), d’Herrelle named these invisible agents “bacteriophages”, quite literally, “eaters of bacteria” (D, Herrelle, 1917).

After his research with dysentery, he moved to France, where he studied avian typhosis. He demonstrated that phage-treated flocks had fewer deaths, shorter epidemics, and fewer recurrences of disease (d’Herelle, 1921). In the early 1920s he treated bovine hemorrhagic septicemia in Indochina (d’Herelle, 1926). In that case, parenteral inoculation of specific phage protected water buffaloes from experimental infections. D’Herrelle also experimented with phage therapy of human diseases, treating bacillary dysentery and four cases of bubonic plague, and also initiating a large-scale trial to reduce cholera infections in India (Summers, 1999).

The success of d’Herelle’s work garnered much attention, and sparked a great deal of interest in phage therapy. However, not all phage treatments were successful, and the image of phage therapy was being tarnished by the sale of unproven remedies in the United States. When

antibiotics were introduced just before World War II, phages were relegated to the sidelines as a therapeutic tool. Penicillin and other antibiotics offered an effective broad-spectrum therapy that was easy to mass-produce, whereas phages were pathogen-specific. Instead, phages became the star tool of early molecular biology and genetics research since their small genomes facilitated the study of basic cellular processes (Summers, 1999).

Limited work on phage therapy continued in North America and Western Europe, including two large cholera studies in Pakistan that were sponsored by the World Health Organization in the 1960s (Monsur et al., 1970; Marcuk et al., 1971). However, these studies are notable because of their rarity, and research interest in phage therapy waned as promising studies were counteracted by reports of failed experiments and by general skepticism about the practical applicability of the idea.

In contrast, research and active use of phage therapy continued in a few parts of Europe during World War II, and in several Eastern Bloc nations afterwards. In the 1920s, Josef Stalin had enthusiastically allowed Georgyi Eliava and Felix d'Herrelle to found an institute of phage research in Tblisi, in the Soviet Republic of Georgia (Summers, 1999). Much of the work conducted in humans has occurred there, but most has not involved double-blind, placebo-controlled trials, or has only been published in Russian or Polish. However, most of the patients who are referred to the phage therapy clinicians at Eliava have chronic or antibiotic-resistant infections that have proven untreatable by every other method available, and have been told that they have less than a 5% chance of recovery. Yet the recovery rate among these patients is a stunning 80% or better if they receive phage treatment at Eliava (reviewed by Sulakvelidze & Kutter, 2005). Clearly, phage therapies can be effective, and yet they have rarely been developed

in other parts of the world, and are virtually unknown in agriculture.

Phages have several properties that make them valuable as therapeutic agents. The mechanisms by which they infect and kill bacteria are unrelated to the mechanisms by which antibiotics act, which means that phages can be used successfully against multi-drug resistant bacteria (Chanishvili et al., 2001). They do not have toxic side-effects in the organism suffering from bacterial infection. Under certain circumstance phages can elicit an immune response in treated individuals, but the response is usually subclinical, and at least in humans, does not often alter the outcome of treatment (reviewed by Sulakvelidze & Kutter, 2005). Phages are specific to one or a few related target bacterial species, and therefore do not affect either eukaryotic cells or the normal bacterial ecology of the surrounding environment. They are both self-replicating and self-limiting; a single application can initiate a population that will flourish so long as a susceptible target is present, but they tend to be rapidly inactivated in the absence of a susceptible host and so do not persist in a non-therapeutic environment (Inchley, 1969; Geier, Trigg, & Merrill, 1973). Because of this close association with bacterial host cells, they are often able to penetrate tissues that are not accessible to chemical or other treatments (Bogovazova, Voroshilova, & Bondarenko, 1991; Bogovazova et al., 1992, both as cited in Sulakvelidze & Kutter, 2005). Finally, their production is inexpensive and does not require complex technologies.

Unfortunately, some of these same characteristics can complicate the practical applications of phage therapy and have been presented as reasons why phages should not be used to control phytopathogenic bacteria. A review by Vidaver (1976) presents the most common of these arguments. She notes that bacteria can become resistant to phages, whether by lysogeny or

mutation, and that phages can act as genetic vectors, facilitating the spread of virulence factors or other genes among a bacterial population by generalized transduction or lysogenic conversion. She also points to the fact that phage replication is highly sensitive to environmental conditions.

The transfer of virulence genes or other undesirable traits between bacteria can be minimized by only using phages that are obligately lytic, and by screening for generalized transduction of molecular markers by those phages. While it is impossible to prove that transduction will never occur, it is possible to quantify a maximum risk. The use of phage mixtures should also help minimize the likelihood of transduction, since it is unlikely that all phages in a mixture would be capable of transduction following careful screening. If transduction did occur, the hypothetical transductant cell should still be lysed by at least one of the other phages in the mixture, preventing its propagation.

Phage mixtures are recommended for any phage therapy because the combination of many carefully selected phages drastically increases the likelihood that the pathogen strains endemic to a particular region will be susceptible to the biopesticide. In fact, Stewart (2001) suggests that the tendency towards single-strain biopesticides is one of the most important and most easily rectified barriers standing in the way of consistent performance by biological control agents in general. The use of phage mixtures also substantially reduces the likelihood of bacteria becoming phage-resistant in the first place (Tanji et al., 2004; 2005).

Finally, while it is true that phages are highly sensitive to environmental conditions, so are their host bacteria. Phages that are effective in the environmental conditions in which the target bacterial species is pathogenic can be selected during phage enrichment and efficacy screening, and their survival in the environment can be enhanced by the addition of protective

chemicals during the production of the commercial product.

These issues should not stand in the way of commercially feasible phage-mediated control of phytopathogens, so long as they are taken into consideration during the process of biopesticide development. Nevertheless, only a few phage therapies have been successfully brought to market. Phages formulated in protective media have been successfully used to control bacterial spot of pepper and tomato caused by *Xanthomonas campestris* (Balogh, 2002; Obradovic & Jones, 2004). This biopesticide is available on a small scale from OmniLytix (Salt Lake City, UT). In addition, a six-phage suspension that reduces contamination by *Listeria monocytogenes* on raw meat products (Carlton et al., 2005; Wagenaar et al., 2005) has been approved by the United States Food and Drug Administration, and is produced by a company called Intralytix (Baltimore, MD).

The key to creating an effective phage-based biopesticide is the philosophy on which the development program is based. The successful development of microbial biopesticides requires that several factors be considered at every stage of research: efficacy, economics, and regulatory constraints. Regulatory requirements include safety/toxicity studies, environmental persistence and impact studies, and aesthetic acceptability of the basic idea to the human beings in charge of the regulatory process. Economic considerations include the ease and cost of production, storage, and use. Finally, and most importantly, the treatment must work. It must predictably control an appropriately broad spectrum of bacterial strains in the field, either reducing the pathogen population to subeconomic levels or removing the pathogen's ability to cause disease. (Murdoch & Briggs, 1996; Schisler & Slininger, 1997; Wilson, 1997b; Stewart, 2001; Ojiambo & Scherm, 2006).



Schisler and Slininger (1997) identified three aspects of initial research that are critical for the development of a commercially feasible biopesticide: “(1) choosing an appropriate pathosystem to investigate; (2) choosing a appropriate method of microbe isolation; (3) conducting an appropriate isolate characterization and performance evaluation.”

The selection of an appropriate disease target is the first step. There must be a bottleneck in the disease cycle, at which point the prevention of transmission or infection will substantially limit further disease development. The factors that limit or permit transmission or infection at this point must be vulnerable to a phage-based treatment in a manner that is consistent with existing treatment infrastructure. In the case of fire blight, open apple and pear blossoms are the primary site of infection. They are present for a finite period of time, and are easily accessible using existing pesticide application technology.

The second step in the development of a biopesticide is the isolation of microbial antagonists. In the case of phages, the isolation of a large number of diverse phages with broad host ranges is facilitated by the use of a multiple host enrichment system (Jensen et al., 1998). This was done by Gill (2000) during the collection of the Vineland phages, and resulted in a collection of *E. amylovora* phages that is much more diverse than any previously reported (Erskine, 1973; Vandeburgh et al., 1985; Ritchie & Klos, 1979; Schnabel & Jones, 2001).

Thirdly, the criteria by which isolates are selected must approximate the criteria which will determine success or failure in the field. A candidate that is highly effective against a laboratory strain in an *in vitro* assay may not be effective against a natural infection. In the case of fire blight research, screening processes are often media-based or use fruit tissue or seedling shoots. Of these, only the seedling assay reflects part of the natural infection cycle, and in none of

these assays do the results reliably correlate with performance on blossoms (Wilson, Epton, & Sigee, 1990; Thibault, & Le Lezec, 1990, as cited by Lespinasse & Aldwinckle, 2000; Wilson, Epton, & Sigee, 1992). Blossom infection is the primary mode of seasonal disease initiation, and so assays based on apple or pear blossoms, such as the one used in this research, are a much more accurate assessment of the efficacy of a biopesticide in controlling *E. amylovora* populations.

The final and most critical step is proving that the biopesticide has high efficacy under field conditions. The complex interaction of biotic and abiotic factors in the orchard can also alter the physiological and biochemical properties of trees in ways that can not be replicated elsewhere. As a result, green-house and growth chamber plants sometimes support higher epiphytic microbial populations (O'Brien & Lindow, 1989; Beattie & Lindow, 1994). The pathogen pressure against which biopesticides are tested must also reflect the natural infection process as much as possible. In many cases, applying a pathogen concentration comparable to indigenous populations will not produce enough disease to obtain statistically significant data unless a impractically large number of sample plants is used (Johnson & Stockwell, 2000). If a higher concentration of pathogen is necessary, care should be taken to ensure that the pathogen population must still increase on the plant surface before infection can take place, otherwise there will be no opportunity for the biopesticide to antagonize the pathogen, as it would in a natural infection setting.

Without a doubt, the greatest hindrance to the development of successful phage therapies has been the absence of an adequate knowledge of the phage-host ecology in the therapeutic environment (Goodridge, 2004; Summers, 2005). The consequence of this has been an historical inability to explain the success or failure of a given treatment. Addressing this issue absolutely

requires that the population dynamics of the biopesticide components and the pathogen be monitored throughout field trials. The effects of environmental stresses on the culturability of bacteria (Wilson & Lindow, 2000) requires that this be done using a culture-independent method.

This research constitutes the first report of an effective, practically feasible phage-based biopesticide for fire blight. The novel strategy developed here uses a non-pathogenic bacterium that is also susceptible to infection by the same phages as a “carrier”. *Pantoea agglomerans* (Ewing & Fife) Gavini et al (formerly *Erwinia herbicola*) is an orchard epiphyte that is closely related to *E. amylovora*. The underlying hypothesis of this work is that the *P. agglomerans* carrier will play a dual role in control of *E. amylovora*, acting directly via competitive exclusion, and indirectly by supporting a replicating population of phages in the hostile blossom environment over the period of time at which the blossoms are at risk for infection. If *E. amylovora* colonizes the blossom, the phages will infect and kill the pathogen such that its population never grows large enough to cause a blossom infection. To accomplish this goal, phages and a carrier will be selected from highly diverse collections of unique *E. amylovora* phages and *P. agglomerans* isolates. These phages are the most morphologically and genetically diverse, and the promiscuous *E. amylovora* phages that have yet been described. The interactions of *E. amylovora* phages, *E. amylovora*, and *P. agglomerans* are extensively studied in both excised blossoms and in the orchard. Multiplex real-time PCR is used here, for the first time, as a means of simultaneously monitoring the population dynamics of the three organisms relevant to the success of the phage-carrier biopesticide - the *E. amylovora* pathogen, *E. amylovora* phages, and the *P. agglomerans* carrier - and thereby make the critical correlation between treatment outcome and microbial ecology.

The research encompassed by this strategy is divided into three parts, outlined below.

## **Part I: A Review of Fire Blight**

### **Chapter 1: Fire blight: The disease and current management strategies.**

A thorough appreciation for the biological context to which a disease control strategy will be applied is fundamental to the development of that strategy. The disease and its causative agent are reviewed here, including distribution, epidemiology, and pathogenesis. Current disease management strategies are emphasized, as this research describes the development of a novel means of fire blight control.

## **Part II: Characterization of Biopesticide Components and Pathogen Growth**

### **Chapter 2: Selection of *Erwinia amylovora* phages and a *Pantoea agglomerans* carrier**

This section describes the performance-based selection and preliminary characterization, of a carrier and the phages that will be the focus of field trials. *In planta* pear blossom assays were used to select carrier and phage isolates that are most likely to have high efficacy under field conditions.

### **Chapter 3: The complete genome sequence of $\phi$ Ea21-4**

The Vineland collection of *E. amylovora* phages displays a tremendous amount of genetic diversity. This diversity has challenged efforts to monitor the population dynamics of these phages. The first complete genome sequence of an *E. amylovora* phage belonging to the *Myoviridae*,  $\phi$ Ea21-4, was sequenced in order to improve understanding of *E.*

*amylovora* phages, and to assist in the continued development of molecular monitoring techniques.

Chapter 4: Real-time PCR reveals competition between *Erwinia amylovora* and *Erwinia pyrifoliae* on pear blossoms.

This work on the Asian pear blight pathogen, *E. pyrifoliae*, and its relationship to *E. amylovora* provided an opportunity to study the growth of *E. amylovora* on blossoms, and to validate some of the protocols being developed to monitor the population dynamics of biopesticide components in the orchard.

### **Part III: Development and Evaluation of the Phage-Carrier Biopesticide**

Chapter 5: Evaluation of the interactions between *Erwinia amylovora*, *Pantoea agglomerans* Eh21-5, and *Erwinia amylovora* phages

An *in planta* assay is used to determine the optimum parameters for field applications of the phage-carrier biopesticide. *In vitro* growth characteristics of *E. amylovora*, *P. agglomerans*, and *Erwinia* phages relevant to the efficient production of the biopesticide components are also studied.

Chapter 6: Detection of *Erwinia amylovora* phages in orchard soil

This chapter describes the development of a method to isolate phages from soil, and to remove soil-derived PCR inhibitors, without the need for a DNA extraction step. This work was undertaken in order to develop a simple and rapid means of tracking the

environmental fate of the phages being used in these experiments.

## Chapter 7: Control of *Erwinia amylovora* with a phage-based biopesticide under field conditions

Phage-carrier combinations are shown to reduce disease incidence by controlling pathogen populations in field trials. Multiplex real-time PCR is used to correlate disease outcome with biopesticide and pathogen population dynamics. Phage production and purification techniques are also described.

## **Part I: A Review of Fire Blight**

## Chapter 1: Fire blight: The disease and current management strategies

### Introduction

Fire blight is a necrotic disease caused by the bacterial phytopathogen *Erwinia amylovora* (Burrill) Winslow et al. The disease affects roseaceous plant species, including *Malus* spp. (apple), *Pyrus* spp. (pear), *Cydonia* (quince), *Cotoneaster* (cotoneaster), *Sorbus* (mountain ash), *Rubus* spp. (raspberry, blackberry), and *Crataegus* (hawthorn). Fire blight has the distinction of being the first disease proven to be caused by a bacterium (Burrill, 1878), and *E. amylovora* was the first phytopathogenic bacterium shown to have insect vectors (Baker, 1971).

Fire blight was first described on apple trees in 1780 in the Hudson Valley of the USA (Denning, 1794), where it probably evolved on local rosaceous species such as hawthorn, mountain ash, and crab apple. The disease spread throughout the United States and Canada during the 19<sup>th</sup> century, and reached New Zealand and England by 1960 (Bonn & van der Zwet, 2000). Fire blight then appeared in the Eastern Mediterranean, and spread quickly through Europe. It is also present in Mexico (Lopez & Fucikovsky, 1990), and unconfirmed observations have been reported in Bermuda, Guatemala, China, Vietnam, and Russia (Bonn & van der Zwet, 2000).

The dissemination of *E. amylovora* has been largely facilitated by human activity. The westward spread of fire blight across North America generally coincided with the movement of European settlers and their establishment of fruit orchards (Bonn & van der Zwet, 2000). Trans-oceanic introductions into New Zealand and England seem to have resulted from the importation of infected nursery stock (Bonn & van der Zwet, 2000). The source of the initial Egyptian



infections is not clear, but the fact that the first report was made near Alexandria, a port city on the Nile delta, makes it likely that this introduction was also related to an agricultural import. The timeline of disease spread suggests that once present in England and Egypt, *E. amylovora* quickly spread to the other reporting European and Mediterranean countries via natural means (Bonn & van der Zwet, 2000). In total, the disease has been reported in at least 40 countries, and is economically important in many of them.

The greatest economic impact of fire blight results from the loss of commercial fruit crops, particularly apple and pear. Because outbreaks occur sporadically and with variable severity, it is difficult to estimate the annual impact of fire blight. Nor are the effects limited to a single year, since shoot blight can kill wood that would bear fruit spurs in subsequent years. A 1998 outbreak in Washington and Oregon caused an estimated \$68 million loss in apple and pear crops (Smith, 1997). The 2000 outbreak of fire blight in southern Michigan caused an immediate crop loss of \$9.7 million, a tree loss of \$9 million, and an estimated total loss of \$42 million over five years until damaged trees recovered their full fruit-bearing capacity (Longstroth, 2000). Outbreaks tend to be more frequent and more severe in warm, humid regions (Bonn & van der Zwet, 2000) where the temperature and moisture conditions that favour pathogen dissemination and infection coincide with the most susceptible host phenology.

### ***Erwinia amylovora*, the Fire Blight Pathogen**

*E. amylovora* is a member of the *Enterobacteriaceae*, the Gram-negative, motile, facultatively anaerobic, non-sporulating bacilli. The organism was originally named *Bacillus amylovorus* (Burrill, 1878). Ninety years ago, all of the phytopathogenic *Enterobacteriaceae*

were classified in the genus *Erwinia* (Winslow et al., 1917). That genus was subsequently divided into the “amylovora”, “carotovora”, “herbicola”, and “atypical” groups based on metabolic characteristics and the type of disease they cause (Dye, 1968; Dye, 1969a; Dye, 1969b; Dye, 1969c). DNA:DNA hybridization studies and rDNA sequence analyses led to further taxonomic reorganization of these organisms, with most of Dye’s herbicola group and some of the atypical group being assigned to the genus *Pantoea* (Ewing & Fife, 1972), most of the carotovora group being assigned to the genus *Pectobacterium* (Lelliot & Dickey, 1984; Hauben et al., 1998), and an additional monophyletic group of species being placed in the genus *Brennaria* (Hauben et al., 1998).

For all the phylogenetic turbulence suffered by current and former members of the *Erwinia* genus, *E. amylovora* is, itself, a remarkably homogeneous species. There is little variability in metabolism or serology, and what variation has been described has not been linked to differences in virulence (Billing et al., 1961; Vantomme et al., 1982; Slade & Tiffin, 1984; Vantomme et al., 1986; Verdonck et al., 1987). Traits of note include a strict requirement for nicotinic acid, which is not common among the Enterobacteriaceae, and the ability to use both sucrose and sorbitol as carbon sources, since these are the main forms of carbon reserves in apple and pear (Bielecki, 1969).

There are no recognized pathovars or biovars of *E. amylovora*, and most strains are not species-specific. Those isolated from Maloidae (ie. apples, pears, hawthorn, and quince) are pathogenic on Amygdaloidae (ie. Japanese plum), Rosoidae (ie. raspberry), and other Maloidae. The one exception seems to be isolates from *Rubus* spp., which are not pathogenic on apple or pear (Starr, Cardona, & Folsom, 1951; Ries & Otterbacher, 1977; Heimann & Worf, 1985).

Some genetic markers distinguish these isolates, but the biological basis of their different host specificity is unknown (Laby & Beer, 1992; McManus & Jones, 1995b; Jock & Geider, 2004; Giorgi & Schotichini, 2005).

In 1903 Uyeda reported fire blight on apple trees in Japan. He identified the pathogen as *Bacillus amylovorus*, and believed that it been introduced on infected nursery stock from the United States (Uyeda, 1903, as cited by Bonn & van der Zwet, 2000). Similar reports of outbreaks on apple and pear were made in the 1920s, and in 1955, a report on bacterial phytopathogens present in Japan included a pear disease caused by an unidentified species of *Erwinia* (Okabe & Goto, 1955, as cited by Bonn & van der Zwet, 2000). In 1992, a report on “bacterial shoot blight of pear” (BSBP) described symptoms very similar to fire blight and a causative agent that was almost, but not quite, identical to *E. amylovora*. The precise identity of this organism became the subject of some debate. Recent studies have presented evidence that the Japanese *Erwinia* isolates are much more closely related to, and may in fact be, *Erwinia pyrifoliae* (Kim et al., 2001a; Matsuura et al., 2007; Chapter 4).

Given the historical reports of fire blight-like disease in Japan, and the pattern of similarities between *E. amylovora*, the Japanese *Erwinia* isolates, and *E. pyrifoliae*, one possibility is that *E. amylovora* was imported into Japan on nursery stock in the early 1900s, and then evolved through a combination of genetic drift and adaptation to local host species, particularly *Pyrus pyrifolia*, as it spread through Japan, eventually reaching Korea. But where gene flow between *E. amylovora* populations within North America and Europe likely continues to this day, the population imported into Japan 100 years ago would have been geographically isolated, setting the stage for speciation. As a result, the “newest” species is *E. pyrifoliae*, found

in Korea, and the Japanese *Erwinia* isolates are much more similar to that species than they are to *E. amylovora*. The constant occurrence of local adaption is supported by the gradual spread of *E. amylovora* from highly susceptible pears to more resistant apples over the first 10 years in which fire blight is reported in a new region (van der Zwet & Keil, 1979), and by regional and stress-related microdiversity within the short-sequence repeat (SSR) region of plasmid pEa29 (Jock et al., 2003a). Certain similarities between the SSR regions of *E. amylovora* and the Japanese *Erwinia* strains also suggest that these two organisms shared a single, common ancestor.

It has been argued that “bacterial shoot blight of pear” (BSBP) is identical to fire blight, regardless of the host specificity or bacterial strain involved (Beer et al., 1996). There is substantial merit to this argument. In medicine, the term “disease” refers to pathology, the set of symptoms that a patient presents. Frequently, that disease may be caused by one of several different etiologic agents. In plant pathology, however, the general practice is to define a disease by the specific identity of its causative agent. If this is the standard to be used then it must be conceded that Asian pear blight, and most likely BSBP, are not, strictly speaking, fire blight. However, this perspective must not be allowed to overshadow the practical implications of disease management, or the potential insight into phytopathogen evolution that could be provided by more detailed phylogenetic studies of these *Erwinia* species.

## **The Disease Cycle**

The nature of a disease cycle is that it has no objectively definable beginning or ending. However, from the perspective of the commercial grower, the fire blight disease cycle begins anew each spring. There are five generally accepted types of fire blight: blossom blight, shoot

blight, canker blight, rootstock blight, and trauma blight (Steiner, 2000). Each is characterized by a distinct set of symptoms, and the location of the *E. amylovora* population that is responsible.

Figure 1-1 shows the progressions of infection and symptom development that have been observed. Primary infection generally takes the form of blossom blight. *E. amylovora* cells that have survived the winter in canker margins replicate and form a bacteria-rich “ooze” that is disseminated by wind, rain, and pollinating insects. The bacteria will then colonize the blossoms that are just beginning to open (Miller, 1929). The young blossom (Figure 1-2), and the stigma in particular, offers a very favourable environment for the growth of *E. amylovora* (Thomson, 1986; Wilson, Epton, & Sigee, 1989). It is a moist, sugar-rich environment amid the comparatively dry orchard canopy, and there is little competition from other epiphytic bacteria when the blossom first opens (Stockwell et al., 1999). Bacteria can then easily spread from blossom to blossom by foraging pollinators (van Leare, de Greef, & de Wael, 1982; Thomson et al., 1992; Johnson et al., 1993a; Thomson, Wagner, & Gouk, 1998). The actual infection of the blossom occurs through natural openings in the hypanthium, when bacterial cells that have accumulated in the stigmatic secretions are washed into the hypanthium by rain or dew (Thomson, 1986; Wilson, Epton & Sigee, 1989; Pusey, 1997).

The susceptibility of individual blossoms seems to be greatest within a few days of opening, and then declines, becoming resistant once petal fall commences (Hildebrand & Heinicke, 1937; Gouk, Bedford, & Hutshins, 1996; Thomson & Gouk, 2003). It should be noted however, that most of these assessments are based on studies of bacterial growth, rather than on direct assessments of the susceptibility of hypanthial tissues.

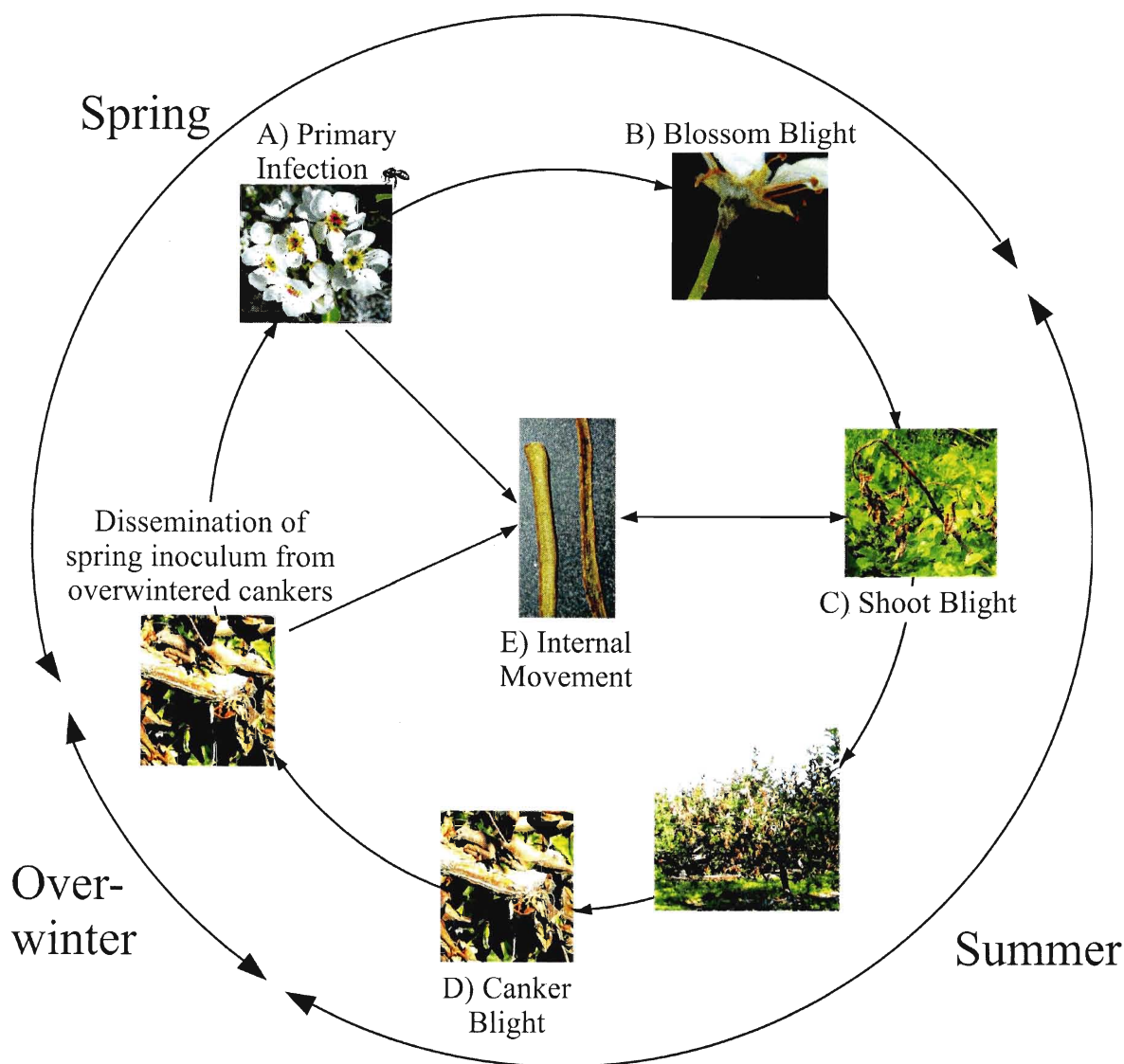


Figure 1-1. The fire blight disease cycle. A) Young blossoms are colonized with *E. amylovora*; B) Blossoms become necrotic and release ooze; C) Shoots become necrotic as *E. amylovora* grows intercellularly from the point of primary blossom infection, or from secondary infection due to insects feeding on new succulent tissue, or from direct internal movement of bacteria from overwintered cankers; D) Cankers develop from bacteria that have migrated from primary or secondary infection sites; E) Necrosis extends through the interior of an infected shoot due to internal movement of *E. amylovora* (right). Trauma blight not shown in this figure. (Photos by S. M. Lehman and E. S. Barszcz)

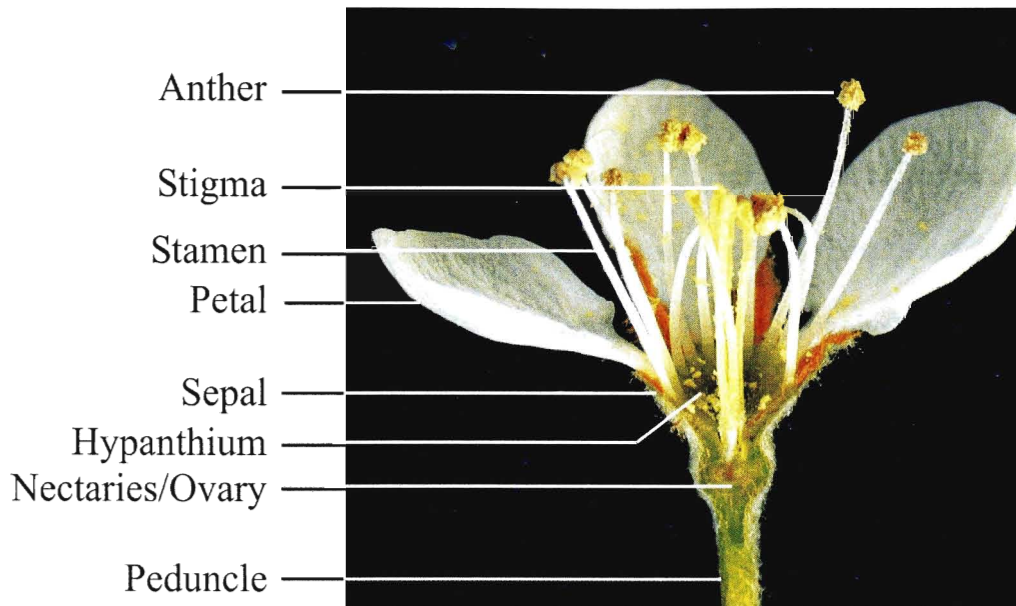


Figure 1-2. Structure of a Bartlett pear blossom. [Photo: S.M. Lehman]

The pathogen continues to multiply as it invades the intercellular spaces of the host. The infected blossom appears water-soaked, then will discolour and become necrotic as cells undergo plasmolysis (van der Zwet & Keil, 1979). The bacteria continue to travel through intercellular spaces, invading the peduncle, the spur, and then moving into the stem. Infected tissues will first appear water-soaked, then wilt and turn brown-black in colour. Infected shoots take on a characteristic “Shepherd’s crook” shape. As the bacteria invade the host tissue they tend to enter xylem vessels (Suhayda & Goodman, 1981; Bogs et al., 1998). Exopolysaccharide (EPS) accumulation is believed to disrupt water flow in the xylem and lead to the formation of bacterial aggregates (Sjulin & Beer, 1978; van Alfen & Allard-Turner, 1979). It is not known whether these aggregates are assemblages of planktonic cells or if *E. amylovora* forms biofilms in a manner comparable to the grape pathogen, *Xylella fastidiosa* (Marques et al., 2002; Newman et al., 2003). *E. amylovora* biofilms have not been observed, but the *rcs* genes that are implicated in the transition between the planktonic and biofilm lifestyles are only beginning to be studied in this organism (Pristovšek et al., 2003; Prüß et al., 2006). Regardless of their form, these aggregates cause the xylem vessels to leak, and an exudate of bacterial cells and EPS is forced to the surface, producing the ooze that is characteristic of fire blight. This ooze contains virulent *E. amylovora* cells that are easily spread by rain and insects (Miller, 1929; Eden-Green, 1972). The ooze can also dry into long, thin strands that can be dispersed by wind (Bauske, 1971), and easily rehydrated to release virulent bacteria (Eden-Green & Billing, 1972; Keil & van der Zwet, 1972a).

Shoot blight refers to the necrosis of vegetative shoots due to secondary infection following blossom blight or canker blight. Most vegetative tissue infections result from the



spread of endophytic populations (ie. due to prior blossom blight), or from infections through wounds created by insects feeding on succulent shoot tips (Steiner, 2000). The emergence of susceptible succulent shoots generally coincides with the development of large amounts of bacterial ooze from blossom infections, and with the warm, wet weather that favours its dissemination. In susceptible shoot tissues, fire blight symptoms may progress at rates  $>2.5$  cm/day (Blachinsky et al., 2003), spreading rapidly to woody tissues and killing large parts of mature trees.

Orange-brown cankers develop on infected leaders and trunks due to the collapse of the cortex parenchyma (van der Zwet & Keil, 1979). In both pear and apple, cankers that form late in the season are more likely to have indeterminate margins that harbour overwintering populations of *E. amylovora*, and become major sources of inoculum for the following season (Beer & Norelli, 1977; Biggs, 1994). Cankers on other rosaceous hosts nearby can also provide inoculum for the following season (Billing, 1980).

Rootstock blight occurs in apples when *E. amylovora* travels through healthy scion tissue into the rootstock, where it causes cankers that can girdle and kill the tree. It is most common and most lethal when susceptible scions are grafted on to highly susceptible M.9 and M.26 rootstocks (van der Zwet & Beer, 1995; Steiner, 2000), but resistant scions do not necessarily protect susceptible rootstocks (Suleman & Steiner, 1994). Rootstocks can also become infected through suckers or water sprouts. It is not known why some trees develop rootstock blight while others do not, but the condition causes an average of 25% of apple trees in the Appalachian region of the USA to die within five years of planting (Steiner, 2000). Loss estimates suggest that this could total almost \$9000 per acre over that five year period (Norelli et al, 2000).

Trauma blight refers to necrosis that results from direct infection of wounds caused by external factors such as hail, frost, and severe winds. Even normally resistant cultivars can become infected when the bacteria have direct access to the xylem through the wound site (Eden-Green, 1972). The occurrence of trauma blight is largely independent of disease incidence at other points in the growing season since inoculum may be present in the orchard without causing disease through any of the previously described mechanisms.

The optimum growth temperature of *E. amylovora* is 25 to 27°C (Billing E. et al., 1961), but it is able to grow between about 5 to 37°C. A substantial change in the relationship between doubling time and temperature has been reported for temperatures above (versus below) 18°C, both in pure culture and in apple shoots, suggesting that temperatures above 18°C are significant to disease development in the orchard (Billing, 1974).

Several studies describe the detection of *E. amylovora*, and even the isolation of virulent cells, in asymptomatic tissue (Baldwin & Goodman, 1963; Keil & van der Zwet, 1972b; McManus & Jones, 1995a). Asymptomatic infections may play an important role in the long-term ecology of *E. amylovora*, and in the spread of the pathogen via nursery stock (Calzolari et al., 1982; van der Zwet & Walter, 1996), but the mechanisms that trigger this switch from asymptomatic to an active infections are unknown.

## Pathogenesis

The pathogenicity of *E. amylovora* arises from five factors: bacterial EPS, the hypersensitive response (HR) and pathogenicity gene cluster (*hrp*), the disease-specific gene cluster (*dsp*), and the capacity to thrive in the host environment. Unlike the closely related

*Pectobacterium* species, formerly the soft-rot *Erwinias*, *E. amylovora* does not degrade the cell walls of the plant host, as it does not produce any pectinolytic or cellulolytic enzymes (Seemuller & Beer, 1976).

*E. amylovora* produces two exopolysaccharides, amylovoran and levan, that form a loose capsule around the bacterial cells. This composite EPS capsule is a main component of the ooze that exudes from diseased plant tissue (Bennett & Billing, 1978). EPS encapsulation also prevents agglutination by the apple factor that interacts with *E. amylovora* LPS (Romeiro, Karr, & Goodman, 1981a; 1981b), and helps cells retain water and nutrients in dry environments (Jock, Langlotz, & Geider, 2005). Amylovoran is the principle component of the EPS capsule. It is a heteropolymer that consists mostly of galactose and glucaronic acid residues (Nimtz et al., 1996). Its synthesis requires 12 genes on the *ams* operon and is influenced by temperature, pH, and nutrient availability (Geider, 2000). Levan is produced in the presence of sucrose. The secreted levansucrase enzyme (*lsc*) cleaves sucrose molecules, releasing glucose and creating the  $\beta$ -2,6-fructofuranan homopolymer known as levan (Gross et al., 1992). Levansucrase secretion is constitutive in some *E. amylovora* isolates, and temperature-dependent in others (Geier & Geider, 1993; Bereswill et al., 1997).

Amylovoran is absolutely required for pathogenicity, while levan deficiency only results in reduced virulence (Steinberger & Beer, 1988; Geider et al., 1993; Bernhard et al., 1996; Tharaud et al., 1997). However, the means by which EPS contributes to disease development is not clear. Restriction of water movement and changes in membrane permeability have been implicated in wilt induction and tissue collapse, but the results are not definitive (Sjulin et al., 1978; Brisset & Paulin, 1992).

The *hrp* gene cluster contributes to pathogenesis in host plants, and induces HR in non-host plants (Steinberger et al., 1988). The cluster contains two independent regulatory pathways, *hrpXY* and *hrpS*, each inducible by specific carbon and nitrogen sources, temperature, water potential, and low pH (Wei, Sneath, & Beer, 1992; Wei, Kim, & Beer, 2000). Both pathways act through the HrpL sigma factor, which activates the secretory and secreted *hrp* genes (Wei & Beer, 1995). At least 12 proteins are secreted into the host plant cells through a type III secretion system (Nissinen et al., 2007), including HrpN and DspA/E. HrpN is the most abundant of the secreted elicitors of HR and SAR, called harpins, but is not clear whether it is absolutely required for pathogenicity (Wei et al., 1992; Barny, 1995). The *dsp* genes are essential for pathogenesis (Gaudriault et al., 1997; Bogdanove et al., 1998), but their mechanism of action is not understood (Meng et al., 2006; Bonasera et al., 2006).

The development of fire blight is also contingent on the ability of the pathogen to thrive within the host. In the case of *E. amylovora*, this means overcoming iron limitation and metabolizing the available carbon sources. In iron-limited environments such as the blossom and the plant intercellular spaces, *E. amylovora* produces iron-chelating desferrioxamine siderophores and cognate receptors associated with the outer membrane (Kachadourian et al., 1996; Dellagi, Reis, Vian, & Expert, 1999). Receptor expression is required for full virulence, but desferrioxamine E deficiency only impairs growth and virulence on blossoms (Dellagi et al., 1998; Dellagi et al., 1999), suggesting that the actual scavenging of iron for later uptake is less problematic within shoot tissue than it is on blossoms. The *srl* regulon and the *scr* operon are responsible for sorbitol and sucrose metabolism, respectively. Sorbitol is the main molecule used for carbohydrate transport (Zimmermann & Ziegler, 1975; Raese, Williams, & Billingsley, 1978;

Grant & Rees, 1981), and sucrose is most abundant in the nectaries of host blossoms (Bieleski, 1969). Since the blossom is an important infection site for *E. amylovora*, and the pathogen multiplies and moves intercellularly, *E. amylovora* must be able to use both of these carbon sources in order to thrive.

Little is known about the determinants of host specificity in *E. amylovora*. The restriction fragment length polymorphisms (RFLPs) that are characteristic of the limited host-range isolates from *Rubus* spp. are not known to be directly related to the causes of host specificity.

Photosynthetic products do not appear to be involved (Braun & Hildebrand, 2005). The only possibility currently suggested relates to the *dsp* gene cluster. It was noted that the four apple genes whose products interact with DspA/E are conserved in the genomes of host plants, but not in several non-host plants (Borejsza-Wysocka et al., 2006). The interactions between DspE and these four proteins seem to be involved in disease development, and this is the only such difference between host and non-host plants that has been reported.

### **Current Disease Management Strategies**

Plant disease is frequently discussed in terms of the disease triangle. Factors associated with the pathogen, the host and the environment all combine to determine the total amount of disease that develops. Environmental considerations include the general climate of the growing region, any microclimate effects produced by local geography, and the day to day weather. Several factors can affect overall host susceptibility, including the intrinsic genetic susceptibility of a particular variety, the nutritional and water status of the planting, planting density, and other cultural practices. The infectiousness of the pathogen is affected by its intrinsic virulence, total

population size, and the presence of suitable vectors.

Fire blight is a prime example of an agricultural disease that can not be controlled by any single method. The complexity of the disease cycle, and the virtual impossibility of eradicating *E. amylovora* once it is present in an orchard force the commercial grower to manage the economic damage from fire blight by suppressing the pathogen population and maximizing host resistance, in the context of the local environment. This is achieved through a combination of orchard management and the timed application of biological or chemical pesticides.

### **Orchard Management**

The risk of fire blight can be substantially reduced by minimizing the amount of *E. amylovora* present in an orchard. Previously infected tissue, including branches, cankers, or whole trees, can be removed during winter dormancy as part of the usual efforts to optimize tree shape and fruiting capacity (Steiner, 2000; Celetti & Carter, 2004; OMAFRA, 2006). This removes a major source of spring inoculum without spreading active populations of *E. amylovora*. Mid-season removal of active infections is sometimes conducted as well, but because bacterial invasion of healthy tissue outpaces symptom development, and because summer pruning can encourage a flush of susceptible growth, this practice can actually increase disease severity (Suleman et al., 1994; Wilson, 2000; Shtienberg et al., 2003).

To a degree, growers can create nutritional conditions that are less favourable for the infection and intercellular spread of *E. amylovora*. As a general rule, cultural conditions that stimulate vigorous growth will increase the incidence and severity of fire blight (van der Zwet & Keil, 1979). The biological basis of this relationship is poorly understood, but may stem from

the pathogen's response to either high sorbitol levels or high rates of flux in sorbitol levels that are observed in highly susceptible, rapidly growing tissues (Bielecki, 1969; Suleman et al., 1994; Li & Li, 2005; Blachinsky et al., 2006).

Regardless of the underlying mechanism, well-drained soils facilitate the development of extensive root systems that provide adequate hydration without supporting excessive vegetative growth, and are generally associated with reduced fire blight susceptibility (van der Zwet & Keil, 1979; Toselli et al., 2002). In addition, extreme nitrogen levels have long been associated with fire blight susceptibility because it favours succulent growth (Nightingale, 1932; Hildebrand et al., 1937; Lewis & Kenworthy, 1962; Keil & Shear, 1972; Aldwinckle & Beer, 1976; van der Zwet & Keil, 1979). Since growers are far more likely to over-fertilize orchards in an attempt to increase fertility than they are to under-fertilize them, orchard management guidelines tend to emphasize the risk of excessive nitrogen application (Bonn & Carter, 2002; OMAFRA, 2006). Mineral nutrition can affect fire blight susceptibility independently of tree vigour. Mineral deficiencies in general have been shown to increase fire blight susceptibility (Aldwinckle et al., 1976), while high foliar levels of calcium, potassium, and some other specific nutrients are associated with a protective effect (Lewis & Kenworthy, 1962; Koseoglu et al., 1996).

In general, growers should strive for uniform, moderate soil fertility, balanced mineral nutrition, and moderate seasonal vegetative growth, while making minimal external inputs. At the very least, sudden, large changes in these factors should be avoided.

## **Genetic Resistance**

Nearly all Maloidae fruit and ornamental cultivars are clonal, and are vegetatively

propagated by grafting desirable cultivar scions onto rootstocks that are selected for the attributes they confer upon the grafted tree. The intrinsic fire blight resistance of a given planting is determined by the particular combination of rootstock and scion cultivar. However, those genetic factors interact heavily with the cultural and environmental influences discussed above. Tissues of different ages and types often show different degrees of susceptibility, and the mode and site of inoculation can also affect disease outcome (Quamme, van der Zwet, & Dirks, 1976). Resistance classifications are therefore relative, fairly general, and subject to some debate, since they are necessarily a compilation of many studies and years of field observation.

Table 1-1 shows the relative susceptibilities of certain apple and pear scion cultivars. Physiology affects these classifications to some extent, with the more vigorous apple cultivars being more susceptible to fire blight, but the underlying genetic basis of cultivar resistance is poorly understood. No monogenic resistance has been characterized in either apple or pear. Breeding experiments and molecular data indicate that the genetic determinants of fire blight resistance are mostly additive, due to quantitative trait loci (Quamme, Kappel, & Hall, 1990; Calenge et al., 2005; Khan et al., 2006). Unfortunately for pesticide reduction initiatives, most popular commercial varieties are moderately or highly susceptible to fire blight. This is particularly true of the fresh market varieties, which are most subject to aesthetic restrictions and changing consumer preferences.

The rootstock onto which a scion cultivar is grafted also affects its growth and disease resistance. The most commonly used apple rootstocks in Canada are M.9 and M.26. They generate productive dwarf trees that thrive in high-density plantings, but are very susceptible to fire blight. The M.7 rootstock confers increased fire blight resistance to grafted scions, but is not



Table 1-1. Relative susceptibilities of commercial apple and pear cultivars to fire blight. Data are compiled from van der Zwet & Beer, 1995, Babadoost, 2005 and Beckerman, 2007

Apples		Pears	
Highly Susceptible	Ambrosia, Braeburn, Cortland, Fuji, Gala, Idared, Jonathon, Liberty, Mutsu (Crispin), Paulared, Pink Lady, Rome Beauty, Russet	Highly Susceptible	Anjou, Bartlett, Bosc, Comice, Flemish Beauty
Moderately Susceptible	Empire, Golden Delicious, Granny Smith, Honeycrisp, Jonagold, McIntosh, Northern Spy, Spartan	Moderately Susceptible	Asian pears Hosui and Shinseiki
Moderately Resistant	Red Delicious	Moderately Resistant	Keiffer, Old Home

commonly used because it does not have a dwarfing effect (Cline et al., 2001; Cline, Byl, & Hainstock, 2003). Pear scions are usually grafted onto pear or quince seedling rootstocks, neither of which confers any fire blight resistance on the scion.

The creation of transgenic scion and rootstock tissues that carry monogenic resistance factors has also been investigated, but regulatory and public resistance to genetically modified organisms has hindered further development.

### **Chemical and Biological Control Agents**

Several different classes of chemicals are used to reduce fire blight-related crop losses. Copper compounds are used prior to bloom, antibiotics and bacterial antagonists are used to reduce blossom populations of *E. amylovora*, plant growth regulators are used to minimize shoot blight, and inducers of systemic acquired resistance are intended to elicit a general protective effect. Of these, copper, streptomycin, bacterial antagonists, and the plant growth regulator prohexadione-calcium, are registered in Canada, and only streptomycin and bacterial antagonists can directly prevent blossom blight (OMAFRA, 2007).

#### *Antibiotics*

Streptomycin is the bactericide of choice for controlling *E. amylovora* populations. It is an aminoglycoside antibiotic, and acts by binding to the 30S ribosomal subunit, leading to translation errors and an overall inhibition of protein synthesis (Chemotherapeutic agents, 1994). It is highly effective against blossom blight, and is sometimes also used late in the season to prevent trauma blight following severe weather damage.

Mounting concerns about antibiotic resistant bacteria in general, and streptomycin-resistant *E. amylovora* in particular, have led to substantial restrictions on streptomycin use by Health Canada and the regulatory bodies of several other nations. In general, antibiotics are not registered for regular use in the European Union, though they may be used in some countries under special circumstances. Streptomycin resistance has been most problematic in the United States, particularly in Michigan and the northwest coast. Resistance problems in Israel have led to a drastic reduction in streptomycin use in that region, and increased interest in alternative bactericides (Manulis et al., 1998; Shtienberg et al., 2001). Streptomycin-resistant isolates of *E. amylovora* have been found in British Columbia (Sholberg et al., 2001) and New Zealand (Thomson et al., 1993; Vanneste & Voyle, 1998), but the existence of these isolates has not resulted in streptomycin resistant outbreaks of fire blight.

In the United States, epidemic outbreaks of streptomycin resistant *E. amylovora* have generally been associated with excessive use of the antibiotic (Johnson et al., 1998). Drastic reductions in streptomycin use can substantially reduce the proportion of resistant *E. amylovora* in the local population (Schroth, Thomson, & Moller, 1979; McManus & Jones, 1994; Manulis et al., 2003), but the resistant population persists in the absence of selective pressure, and can rebound rapidly if streptomycin use is resumed (Loper et al., 1991; McManus & Jones, 1994). The risk of resistant *E. amylovora* outbreaks can be substantially minimized through the use of infection forecasting models. MaryBlyt (Steiner & Lightner, 1996) and Cougarblight (Smith, 1996) both use information about wetting events, temperature, and bloom stage to predict when an infection is likely to occur, assuming the presence of *E. amylovora*. In Canada, up to three applications of streptomycin are permitted each year. With proper use of these programs, growers

become confident that they are optimizing their allowed streptomycin applications. As a result, they are less likely to place gratuitous selective pressure on the balance between susceptible and resistant *E. amylovora* populations.

Other antibiotics have been registered or tested for fire blight control outside of Canada. Oxytetracycline, formulated as a calcium or hydrochloride complex, is registered in parts of the United States for use on pear and can also be used on apple in Mexico and some US states where streptomycin resistance has become a problem (McManus et al., 2002). Two quinolone antibiotics, flumequine (marketed as Firestop®) and oxolinic acid, can prevent blossom blight as effectively as streptomycin (Dimova, 1990; Brisset et al., 1990; Tsiantos J & Psallidas P, 1993; Aldwinckle, Bhaskara, & Norelli, 2002; Manulis et al., 2003). They are only registered in parts of Europe and Israel, respectively, but are not widely used in these regions because of the prohibitive cost (Hartman et al., 2000; Manulis et al., 2003). Kasugamycin, or kasumin, is another aminoglycoside antibiotic, but its efficacy against *E. amylovora* is variable and has not been widely tested (Aldwinckle & Norelli, 1990; Shtienberg et al., 2001). Streptomycin remains the only chemical currently registered for blossom blight prevention in Canada. This registration is under constant review and may be withdrawn with little notice, leaving Canadian growers without a reliable method of controlling *E. amylovora*.

### *Plant Growth Regulators*

Prohexadione-calcium (marketed as Apogee®), is available in Canada and the United States for the prevention of fire blight in apples. Apogee is not useful for preventing blossom blight as it has no bactericidal effect. Instead, it is applied during late bloom or early petal fall in

order to limit the growth rate, and thus the susceptibility, of the current year's vegetative growth (OMAFRA, 2006). The dosage of Apogee must be adjusted according to the vigour of a particular cultivar, so as not to excessively suppress vegetative growth in a moderately or minimally vigorous graft-scion combination. In some cultivars, chemical growth regulation, much like mid-season pruning, can induce secondary or "late-season" bloom, creating a second risk period for blossom blight (Deckers, Faust, & Miller, 1992). However, when applied properly, Apogee can be effective against shoot blight in mature apple trees (Aldwinckle et al., 2002).

### *Copper*

The use of copper as a prophylactic treatment for fire blight began in the early twentieth century, following the development of Bordeaux mixture in France (Sutton, 1996). More recently, "fixed copper" formulations have been developed that contain complex copper sulphates, oxychlorides, and oxides (OMAFRA, 2006). The activity of copper compounds is largely attributed to free copper ions, which disrupt cell membranes and protein function by inactivating enzymes and structural proteins (Disinfectants and antiseptics, 1994). Unfortunately, it is not only orchard microorganisms that are affected; vegetative and reproductive plant tissues are also susceptible. The phytotoxic effects of copper compounds generally limit their application to early spring when only woody tissue is present.

Copper compounds appear to be an economically attractive treatment, since they are a relatively inexpensive pesticide. However, the efficacy of copper as a means of preventing fire blight is debatable. When Bordeaux mixture was first introduced, two to four applications per

season were common. The true impact of these treatments on fire blight is not clear, but apple growers still experienced significant disease-related losses (Sutton, 1996). Controlled studies of modern copper-based products are highly variable. Some have reported disease control comparable to that afforded by streptomycin (Aldwinckle et al., 2002), but most reveal little to no efficacy (El Nasr, Hamdy, & Ali, 1990; Dimova, 1990; Tsiantos et al., 2003) or efficacy only when a high concentration is applied (Tsiantos et al., 1993) or when inoculum pressure is low (Tsiantos et al., 2003).

Advocates for copper use stress the importance of thoroughly covering exposed bark and buds in order to create an inhibitory barrier that will prevent *E. amylovora* from colonizing those surfaces (Steiner, 1992; Steiner, 2000; OMAFRA, 2006). Bacteria residing in canker margins will not be eradicated, and ooze from cankers will penetrate the residue to release live bacteria. It is just that any dispersed bacteria should not be able to colonize the rest of the orchard while the copper residue persists. With this in mind, the lack of definitive results from efficacy studies may be attributable to two factors: the timing of copper application relative to pathogen activity, and the choice of where to apply it. Advocacy of green-tip copper applications is predicated on the belief that substantial movement of *E. amylovora* through the orchard begins at this stage of growth (Steiner, 2000). In cool climates such as southern Ontario, the temperatures that favour ooze production and active insect vectors are not generally achieved until trees begin to bloom. At this point, residual copper levels may be insufficient to affect bacterial colonization, and would still be irrelevant insofar as blossom colonization is concerned. Even if copper is present in sufficient quantities, it can only have an effect if all potential colonization sites in the vicinity of the orchard are treated. This includes non-susceptible cultivars that could otherwise harbour

viable bacteria even while remaining unaffected (Steiner, 2000; OMAFRA, 2006).

### *Biological Control*

The purpose of biological control is to reduce the abundance of a particular pest or pathogen by exploiting the ecological interactions between that organism and its enemies or competitors. Ideally, the active agent in a biopesticide is a species that is endemic to the region in which it will be used. This removes the issue of exotic species introductions from the regulatory process and simplifies environmental impact assessments. From a practical standpoint, however, this criterion limits the development of commercial biopesticides. Geographic differences in species diversity make it difficult to find a species that is endemic to enough areas to make it profitable to develop. To date, five commercial biopesticides are available for fire blight management, all of which consist of a single, lyophilized bacterial species. Nufarm Agricultural Inc. produces two products under the BlightBan™ name. BlightBan A506 contains *Pseudomonas fluorescens* A506, and BlightBan C9-1 contains *Pantoea agglomerans* C9-1. Bloomtime Biological FD (Northwest Agricultural Products) and Blossom Bless (Gro-Chem New Zealand Limited; marketed as “PomaVita” in Italy) contain *P. agglomerans* E325 and P10c, respectively. *Bacillus subtilis* is the active ingredient in Serenade® Max (AgraQuest, Davis, CA), which was recently registered in Canada. All of these products function to suppress *E. amylovora* populations on susceptible blossoms.

Pre-emptive exclusion is the process by which one organism establishes itself in a particular niche, thereby preventing an organism that arrives later from flourishing. In this case, that involves colonizing blossom surfaces and beginning to utilize the available nutritional

resources prior to the arrival of *E. amylovora*. Applied alone, the efficacies of BlightBan A506 and Serenade depend entirely upon this process (Wilson & Lindow, 1993), whereas *P. agglomerans* can also suppress *E. amylovora* populations by antibiosis, the antagonism of one organism by the metabolites of another (Stockwell et al., 2002; Giddens, Houliston, & Mahanty, 2003). A diverse range of antibiotics are produced by many strains of *P. agglomerans* (Ishimaru, Klos, & Brubaker, 1988; Wodzinski & Paulin, 1994; Kearns & Mahanty, 1998; Jin et al., 2003), the best described of which are herbicolin O and I (or pantocin A and B, respectively), the two antibiotics produced by *P. agglomerans* C9-1. The Bloomtime Biological FD strain reportedly produces antibiotics, but it is not known whether they are active on blossoms (Pusey, 2002). It now appears that *P. fluorescens* A506 is capable of antibiosis on blossoms, but this activity, and the concomitant increase in disease control, requires the co-application of biologically available iron (Temple et al., 2006).

The efficacy of bacterial antagonists is inherently more variable than that of streptomycin. BlightBan A506 reduces the incidence of fire blight symptoms by 30% to 70%, and BlightBan C9-1 by 50% to 80% (Johnson et al., 1998). Little data is available for either Bloomtime Biological FD or Blossom Bless, but their efficacy appears to be similarly variable (Pusey, 2002; Vanneste, Cornish, Yu, & Voyle, 2002; Werner, Heidenreich, & Aldwinckle, 2004). Some of this variability is attributable to inconsistent blossom colonization. The use of lyophilized cells, as in commercial product formulations, results in more reliable establishment of bacterial populations greater than the  $1 \times 10^4$  to  $1 \times 10^6$  CFU/blossom that is required for successful inhibition of *E. amylovora* (Johnson et al., 1993a; Stockwell et al., 1998). The generally greater efficacy of *P. agglomerans* relative to other species is attributed to their capacity for antibiosis, and their



superior growth and survival on blossom hypanthia (Pusey, 1997; Pusey, 2002). The majority of antagonism between *E. amylovora* and bacterial biopesticides is thought to occur on the stigma, but in order to infect a blossom, the pathogen must ultimately have access to the nectaries via the hypanthium.

Combinations of BlightBan A506 and C9-1 have also been tested. It was thought that the juxtaposition of two organisms with overlapping, but not identical, growth characteristics and mechanisms of action might increase the reliability of fire blight control by creating a more robust bacterial community on the blossom surfaces. Unfortunately, while co-inoculation increased the colonization success of both strains, there was no additive or synergistic effect on disease suppression (Stockwell, Loper, & Johnson, 1992; Nuclo, 1997). It was eventually determined that *P. fluorescens* A506 produces an extracellular protease that inactivates the antibiotics produced by *P. agglomerans* C9-1 (Anderson, Stockwell, & Loper, 2004).

The efficacy of these biocontrol agents depends first upon their ability to colonize open blossoms, and then on their ability to inhibit the growth of *E. amylovora*. Apple and pear blossoms do not usually support detectable populations of bacteria prior to petal expansion, but are then rapidly colonized by a diverse range of species (Stockwell et al., 1999). Young blossoms are also more easily colonized than older ones (Pusey & Curry, 2004). It is therefore recommended that these products be prepared as a suspension of  $1 \times 10^8$  CFU/mL, and applied when orchards are at 15-20% bloom, and again at 75-100% bloom (Johnson & Stockwell, 2000; Agriculture and Agri-Food Canada, 2007). Initially, antagonist populations are about  $1 \times 10^2$  to  $1 \times 10^4$  CFU/blossom (Stockwell, Johnson, & Loper, 1998; Lindow & Suslow, 2003). Populations then increase gradually and spread to blossoms that have opened after the initial treatment

application, aided by warm moderate daytime temperatures and pollinator activity (Johnson et al., 1993a; Nucló et al., 1998; Johnson et al., 2000; Pusey, 2002; Lindow & Suslow, 2003).

There has been some resistance to the registration of these products in the European Union because of the association between organisms in the *Enterobacter agglomerans* complex and human opportunistic infections. The *Enterobacter agglomerans* complex is an extremely diverse collection of strains and species that were previously classified in the genus *Enterobacter* based on biochemical phenotypes. DNA analysis has since revealed that some of these species do not truly belong to the genus *Pantoea*. Of those that do, there is little data available on the differences between clinical and plant-associated strains, though some phenotypic differences have been reported (Lindh et al., 1991). Plant-associated *P. agglomerans*, which is ubiquitous in the environment (Riggle & Klos, 1972; Ishimaru, Klos, & Brubaker, 1988; Grimont & Grimont, 2005), has occasionally been implicated in human infections (Kratz et al., 2003). However, such reports are extremely rare, generally involve immunocompromised or seriously wounded patients, and do not conclusively demonstrate that *P. agglomerans* is the causative agent rather than a benign bystander.

## Summary

Figure 1-4 summarizes the multitude of factors that can affect the fire blight disease cycle. The integration of multiple control products into a pest management program that takes advantage of these factors in a commercial orchard requires some adjustment to usual practices. Forecasting programs exist that can accurately predict peak infection risk periods (Smith, 1996; Steiner & Lightner, 1996), but they must be used differently for biopesticides than for streptomycin. Streptomycin is immediately effective upon application, and active residues will persist for a few days (Smith, Wiens, & Svircev, 2002). In contrast, bacterial antagonists are most effective when applied 48 to 72 h prior to the establishment or growth of significant *E. amylovora* populations (Wilson and Lindow, 1993; Nuclio et al., 1998). As the attempts to combine BlightBan A506 and C9-1 clearly show, the compatibility of different treatments must also be considered. Each of the described biopesticides is naturally resistant to streptomycin, allowing them to be used as part of an integrated pest management program designed to reduce overall antibiotic use without relying completely on less predictable biopesticides. Other pesticides can also have indirect effects on each other. Prohexadione-calcium, which is applied to apples during the latter half of the bloom period to reduce the incidence of shoot blight, alters the composition of nectar, which can in turn affect the growth of bacteria in the blossom (Pusey, 1999; Spinelli et al., 2005).

The prevention of plant disease accounts for a small fraction of total antibiotic use in the developed world, less than 0.5% in the United States (McManus et al., 2002). Nevertheless, the desire to reduce agricultural antibiotic use has become a common theme in regulatory arenas around the world, and is driving determined searches for more ecologically sound alternatives.

However, in the case of fire blight, this research continues to emphasize the superior efficacy of antibiotics and the importance of preventing blossom blight. The efficacy of heavy metals is questionable, the commercial release of transgenic tissue is not yet widely accepted by the public, and the individual efficacy of plant growth regulators, and biological controls is generally less than that of streptomycin. This should not necessarily be interpreted as a failure to produce alternatives to antibiotics. Rather, it is a reflection of the nature of modern integrated pest management, where dependence on single, potent, pesticides is being replaced by a more diversified set of compatible “reduced risk” treatments.

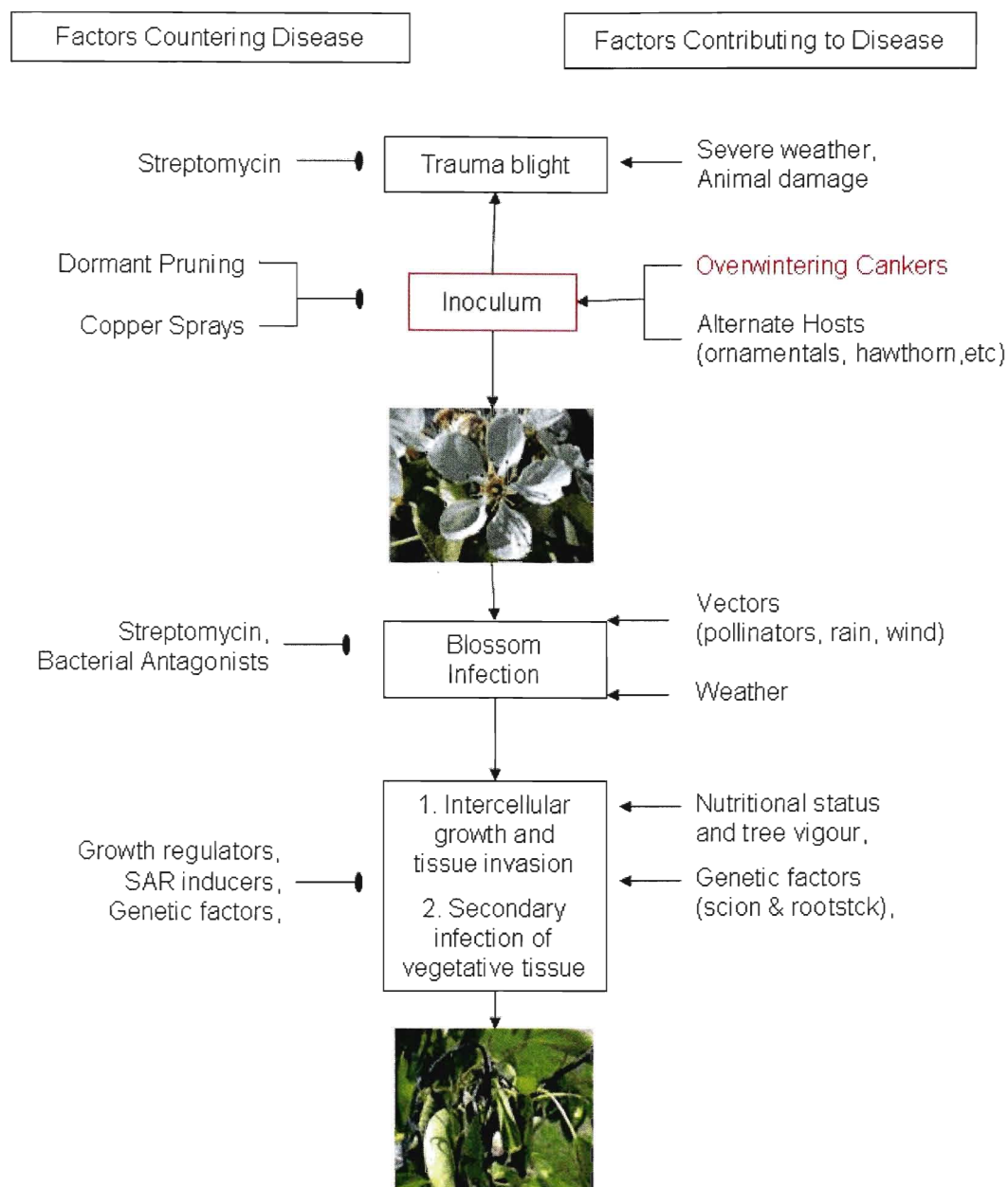


Figure 1-3. Factors affecting the development of fire blight in the orchard. Both Trauma blight and shoot infections can lead to the formation of overwintering cankers, which provide inoculum for the following season.

## **Part II: Characterization of Biopesticide Components and Pathogen Growth**

## Chapter 2. Characterization and screening of bacteriophages and *Pantoea agglomerans* isolates with the potential to inhibit *Erwinia amylovora*.

### Abstract

The components of a phage-based biopesticide for fire blight were selected and characterized in terms of their individual genetic and phenotypic traits, and their interactions. Fifty-six phage isolates and 249 bacterial epiphyte isolates were screened. The genetic diversity of phages was assessed using RFLPs of genomic digests, and by PCR. The *in vitro* susceptibility of *E. amylovora* strains and orchard epiphyte isolates to infection by *E. amylovora* phages was assessed. Biocontrol efficacy was assessed using an *in planta* assay. Pear shoots bearing dormant buds were harvested in late winter, placed in water at 20°C, and allowed to flower. Individual opened blossoms were inoculated with a phage or carrier candidate, and then challenged with the pathogen. Disease symptoms were evaluated after 4 d. Ten phages were selected for further testing based on host ranges, growth characteristics, and genetic diversity. *P. agglomerans* Eh21-5 was selected as the carrier based on *in planta* biocontrol assays, susceptibility to phage infection, species identity and growth characteristics. *P. agglomerans* Eh21-5 produces an antibiotic different from pantocin A that inhibits multiple strains of *E. amylovora*.

## Introduction

The possibility of using *Erwinia* phages to manage fire blight by controlling orchard populations of control *E. amylovora* has been suggested several times (Erskine, 1973; Ritchie, 1978; Schnabel et al., 1998; Schnabel & Jones, 2001; Gill, 2000; Gill et al., 2003). Phages are obligate parasites, completely dependent on the metabolism of the host cell for their replication. Figure 2-1 depicts the two possible modes of phage replication. In the case of tailed phages, the lytic replication process consists of five basic stages: adsorption, penetration, transition to phage-directed metabolism, morphogenesis, and lysis (Guttman, Raya, & Kutter, 2005). The tail fibres or similar structures interact specifically with host cell surface molecules, leading to irreversible adsorption. The cell wall and inner membrane are penetrated, and the phage genome is transferred from the phage head to the host cell, via the tail. The first phage genes are expressed very quickly, and cause the cell's metabolism to switch from host-directed to phage-directed processes. Many copies of the phage genome are then made. The last genes to be expressed are the ones that encode the structural components of the intact phage particle and the proteins that direct virion assembly.

After a certain period of time the phage holin protein interacts with the inner membrane to permit the passage of the endolysin. The endolysin is then able to digest the cell wall, causing cell lysis and the release of a new generation of mature phages from one initial infected cell (Young & Wang, 2005). Each of these progeny can then infect the next susceptible cell it encounters, allowing the phage population to increase exponentially at the expense of the bacterial host population.

Some phages are also capable of lysogenic replication. Under certain conditions, rapid



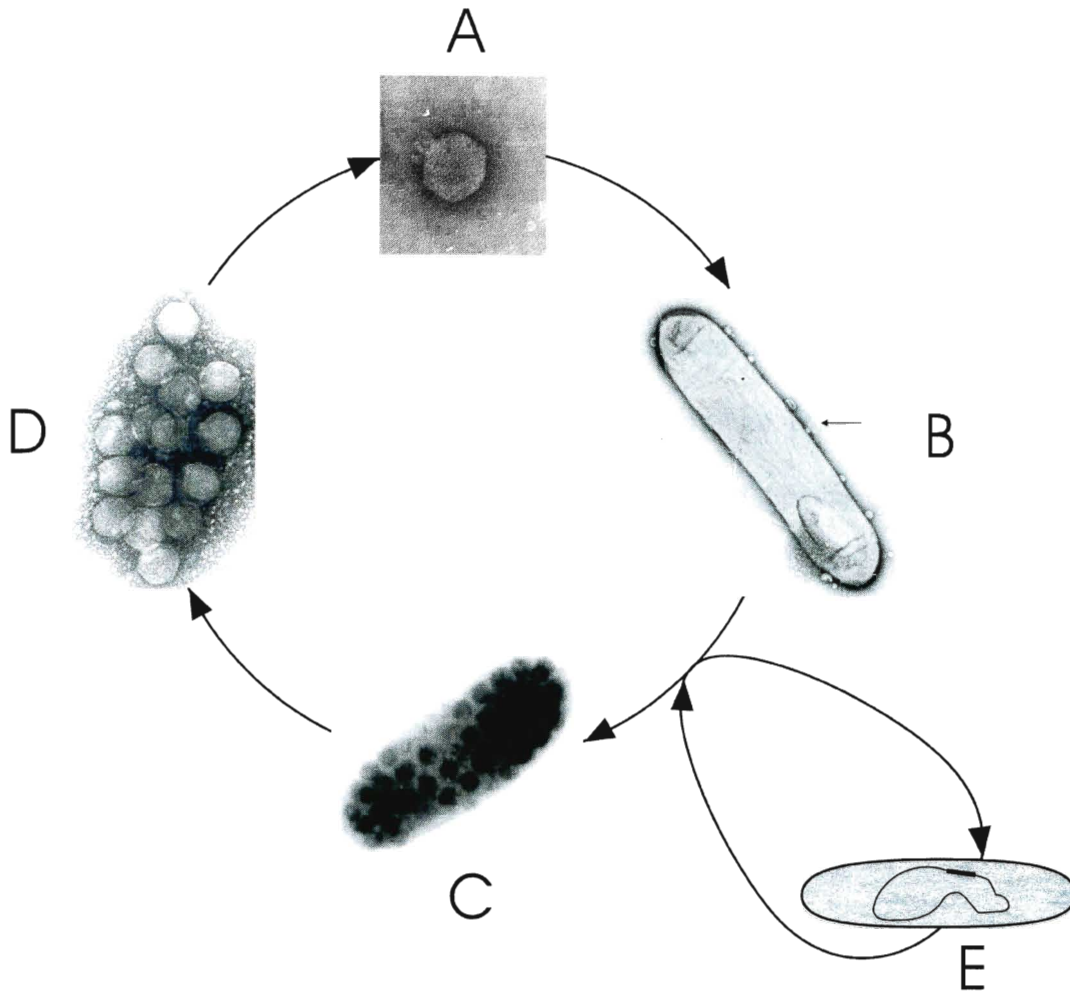


Figure 2-1. The lytic phage replication cycle. A) a single, infective *Podoviridae*; B) irreversible adsorption to an *E. amylovora* cell; C) progeny phage being assembled within the host cell; D) upon lysis, multiple progeny phage are released from a single infected cell; E) a cell in the lysogenic state carries a copy of the phage genome integrated into its own chromosome. [Micrographs A, B, and D are  $\phi$ Ea9-5 and *E. amylovora*, taken by Ronald Smith; C, from Weinbauer & Peduzzi, 1994]

expression of a phage-encoded repressor immediately after genome transfer prevents expression of most phage genes, and promotes recombination between the phage and host genomes. The phage genome becomes integrated into the bacterial chromosome and is passed to all daughter cells in this prophage form. These cells, called lysogens, are also resistant to super-infection by certain other phages, by virtue of the active repressor. Excision of the prophage genome, DNA replication, morphogenesis, and lysis are only triggered when environmental factors interfere with the continued production or activity of the repressor protein. Depending on the precision of the excision process, bacterial genes may be carried along with the phage genome to its next host, where it can become part of that cell's genome. The consequences of this lysogenic replication cycle can include the transfer of pathogenicity or resistance genes throughout a bacterial population.

Bacteria can become resistant to a particular phage independent of lysogeny, usually by altering the surface receptors to which phages adsorb. Since many of these receptors also have important functional roles, that resistance can have adaptive consequences for the bacterial lineage. The development of resistance can be mitigated by using cocktails of phages that interact with different receptors to infect the same cell (Tanji et al., 2004; Tanji et al., 2005). This creates a situation in which the cell could only become resistant in the unlikely event that it acquired mutation(s) that simultaneously conferred resistance to all of the phages in the cocktail.

From this overview of phage biology, it is evident that phages must possess certain distinct traits in order to be useful as a biopesticide. Multiple effective phages are needed, so that they can be applied as a mixture. The selected phages should be exclusively lytic, and most should have broad, but overlapping host ranges.

In order to maintain replicating populations of *E. amylovora* phages on blossom surfaces, those phages must be protected from destruction by ultraviolet light and dessication (Balogh, 2002; Guttman, Raya, & Kutter, 2005; Iriarte et al., 2007). This can be accomplished by formulating a protective suspension medium containing optical brighteners or colloidal suspensions of water-soluble protein (Balogh, 2002). The alternative strategy developed here uses *P. agglomerans*, a non-pathogenic bacterial epiphyte that is also susceptible to infection by the same phages, as a “carrier”. Schnabel et al (1998) reported that *E. amylovora* phage populations declined in the field when applied alone to apple blossoms, but remained high on clusters inoculated with *E. amylovora*, where they reduced fire blight incidence by 26% to 37%. Since virulent *E. amylovora* would obviously not be applied as part of a treatment, the authors suggested that phage survival, and thus disease control, might be enhanced by co-inoculation with avirulent *E. amylovora* mutants. In this, they neglected to consider the potential for reversion of avirulent mutants, and were limited by only considering phages that infected *E. amylovora* rather than those with a slightly broader host range.

The superior exploration of this idea was actually published 25 years earlier when Erskine (1973) reported the discovery of a lysogenic phage capable of infecting both *E. amylovora* and “a yellow, amylovora-like saprophyte” fitting the description of *P. agglomerans*. She noted that none of the disease symptoms associated with *E. amylovora* inoculation appeared when pear fruit slices were co-inoculated with *E. amylovora* and the lysogenized saprophyte, whereas co-inoculation with the unlysogenized saprophyte had only delayed and reduced those symptoms. Erskine ultimately advocated the use of the “phage-infected saprophyte” as a means of biological control of fire blight, correctly noting that phages applied by themselves would be rapidly

inactivated by the ambient conditions, and that the saprophyte would provide some measure of pathogen inhibition on its own. Erskine did not distinguish between the use of bacterial lysogens such as the ones she found, and the use of exclusively lytic phages that happen to infect both bacterial species, as is being proposed in this study. She also supposed that the yellow saprophyte could enter plant tissues to release phages systemically and halt an existing infection, which the non-pathogenic *P. agglomerans* has not been seen to do. However, Erskine did articulate one of the most complete visions of how to put phages to practical use in an agricultural setting.

*P. agglomerans* is a non-pathogenic epiphyte that has long been found in association with *E. amylovora* (Farabee & Lockwood, 1958; Smith & Powell D., 1968; Riggle & Klos, 1972a; Riggle & Klos, 1972b; Erskine & Lopatecki, 1975). Its potential to inhibit *E. amylovora* has also been well-studied. In culture media, some strains of *P. agglomerans* release highly acidic metabolites as they grow, inhibiting *E. amylovora* growth as a result (Wodzinski, Umholtz, Rundle, & Beer, 1994). However, these same strains did not cause a substantial pH change when growing on immature pear fruit tissue, nor was medium acidification in any way associated with a strain's ability to protect pear tissue or apple blossoms from *E. amylovora* infection. Clearly, the *in vitro* behaviour of *P. agglomerans* does not necessarily reflect its interactions with *E. amylovora* in natural infection courts. This is true of antibiotic production as well. Of the 90 antibiotic-producing *P. agglomerans* isolates described by Wodzinski and Paulin (1994), 84 produced antibiotics that were at least partially inactivated by certain amino acids. Strains whose antibiotics are inactivated by the amino acids that are abundant in young pear shoots and in apple and pear fruit did not effectively protect pear tissue from *E. amylovora* infection. In contrast, the antibiotics produced by effective commercial antagonists such as the BlightBan® and

Bloomtime® strains were either unaffected by amino acids or were only affected by those not present in pear tissue.

The selection of suitable phages and a *P. agglomerans* carrier is described here. In 1998, aerial tissue and samples of the surrounding soil were collected from rosaceous plants with active fire blight infections (Gill, 2000). Phages infecting *E. amylovora* were enriched using a mixed culture of six *E. amylovora* strains; 44 isolates were collected. Most of these could be classified into six genetic groups, some of which were also able to infect a few strains of *P. agglomerans*. This phage collection was revived for the current work. The phages were characterized in terms of host range, genetic groupings, and detectability by a real-time PCR. A collection of orchard epiphytes from southern Ontario, consisting largely of *P. agglomerans* isolates, was screened for susceptibility to the Vineland phages and for antibiotic production. The interactions of the phages with bacteria, and of the carrier candidates with *E. amylovora*, were then assessed. The efficacy of the Vineland phages and certain *P. agglomerans* isolates in reducing fire blight symptoms was screened using an *in planta* pear blossom assay. The most promising phages and carrier candidates were selected for use in the biopesticide development program.

## Methods

### *Phage Isolates*

All phage isolates are shown in Table 2-1. Phages were stored in nutrient broth at 4°C unless otherwise indicated.

### *Phage Culture Media and Conditions*

Phages were prepared using either the liquid culture method or the confluent plate lysis method. Unless otherwise indicated, phages were grown on the *E. amylovora* host indicated in Table 2-1.

Liquid cultures were prepared using 8 g/L nutrient broth (Difco Laboratories, Sparks, MD). A 250 mL capped flask containing 50 mL of sterile nutrient broth was inoculated with  $1 \times 10^9$  CFU of the bacterial isolation host, and incubated at 25°C on an orbital shaker at 100 rpm. After 1 h, 1 mL of phage suspension was added to the flask. Flasks were returned to the orbital shaker and incubated for 16-20 h. Chloroform was added to each flask (2%, v/v) and returned to the shaker for 20-60 min. The crude lysate was decanted into 50 mL round-bottom, FEP centrifuge tubes (fluorinated ethylene propylene, Oakridge), leaving the chloroform behind, and centrifuged at 8 000 xg for 25 min. The supernatant was syringe-filtered into sterile 50 mL polypropylene tubes using 0.2 µm surfactant-free cellulose acetate filters (Nalgene, Rochester, NY). Larger liquid cultures were prepared using 500 mL of nutrient broth in 1 L flasks, with proportionately larger bacterial and phage inocula. These lysates were centrifuged in 250 mL polypropylene bottles, and filtered using 0.2 µm GP Express Plus Steri-top filters (Millipore, Billerica, MA ).

Table 2-1. Phage isolates used in this study

Isolate <sup>a</sup>	Isolation Host <sup>b</sup>	Plant source	Source
ΦEa1(h)	Ea110R	symptomatic <i>Malus X domestica</i> tissue	(Schnabel & Jones, 2001a)
ΦEa9-2	Ea17-1-1	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa9-3	Ea29-7	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa9-4	EaG-5	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa9-5	Ea6-4	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa10-1	Ea17-1-1	symptomatic <i>Malus X domestica</i> tissue	AAFC-Vineland
ΦEa10-2	Ea6-4	symptomatic <i>Malus X domestica</i> tissue	AAFC-Vineland
ΦEa10-3	EaD-7	symptomatic <i>Malus X domestica</i> tissue	AAFC-Vineland
ΦEa10-4	EaG-5	symptomatic <i>Malus X domestica</i> tissue	AAFC-Vineland
ΦEa10-5	Ea110	symptomatic <i>Malus X domestica</i> tissue	AAFC-Vineland
ΦEa10-6	Ea29-7	symptomatic <i>Malus X domestica</i> tissue	AAFC-Vineland
ΦEa10-7	Ea29-7	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa10-8	Ea29-7	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa10-9	Ea110	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa10-10	Ea110	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa10-11	Ea17-1-1	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa10-12	EaG-5	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa10-13	Ea6-4	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa10-14	Ea110	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa10-15	Ea110	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa10-16	Ea17-1-1	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa10-17	Ea29-7	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa21-1	Ea110	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa21-2	EaG-5	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa21-3	EaD-7	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa21-4	Ea6-4	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa31-2	EaD-7	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland

Isolate <sup>a</sup>	Isolation Host <sup>b</sup>	Plant source	Source
ΦEa31-3	Ea29-7	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa31-4	Ea29-7	soil beneath symptomatic <i>Malus X domestica</i>	AAFC-Vineland
ΦEa35-2	Ea17-1-1	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa35-3	Ea110	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa35-4	Ea110	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa35-5	EaD-7	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa35-6	EaD-7	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa35-7A ΦEa35-7B ΦEa35-7C ΦEa35-7D	Ea29-7	original ΦEa35-7 was isolated from soil beneath symptomatic <i>Pyrus communis</i> ; source of phages in mixed stock is unknown	AAFC-Vineland
ΦEa45-1A ΦEa45-1B	Ea29-7	original ΦEa45-1 was isolated from symptomatic <i>Pyrus communis</i> tissue; source of phages in mixed stock is unknown	AAFC-Vineland
ΦEa45-3	EaG-5	soil beneath symptomatic <i>Pyrus communis</i>	AAFC-Vineland
ΦEa46-1A1 ΦEa46-1A2 ΦEa46-1A3 ΦEa46-1C ΦEa46-1E	EaD-7	original ΦEa46-1 was isolated from symptomatic <i>Malus sylvestris</i> tissue; source of phages in mixed stock is unknown	AAFC-Vineland
ΦEa46-2	EaD-7	symptomatic <i>Malus sylvestris</i> tissue	AAFC-Vineland
ΦEa50-3	Ea17-1-1	unknown	AAFC-Vineland
ΦEa51-1	Ea17-1-1	soil beneath symptomatic <i>Magnolia sp.</i>	AAFC-Vineland
ΦEa51-2	Ea110	soil beneath symptomatic <i>Magnolia sp.</i>	AAFC-Vineland
ΦEa51-3	Ea6-4	soil beneath symptomatic <i>Magnolia sp.</i>	AAFC-Vineland
ΦEa51-4	Ea29-7	soil beneath symptomatic <i>Magnolia sp.</i>	AAFC-Vineland
ΦEa51-6	Ea6-4	soil beneath symptomatic <i>Magnolia sp.</i>	AAFC-Vineland
ΦEa51-7	Ea29-7	soil beneath symptomatic <i>Magnolia sp.</i>	AAFC-Vineland
ΦEa51-8A ΦEa51-8B ΦEa51-8C	Ea17-1-1 Ea17-1-1 Ea17-1-1	original ΦEa51-8 was isolated from soil beneath symptomatic <i>Magnolia sp.</i> ; source of phages in mixed stock is unknown	AAFC-Vineland

<sup>a</sup> The naming conventions within this phage collection were changed during this work. Previously, all numerical phage designations were prefixed with “PEa”. This prefix has been replaced with “ΦEa”.

<sup>b</sup> The isolation host is the *E. amylovora* strain upon which the phage was originally isolated (Gill, 2000).

<sup>c</sup> The phages in the Vineland collection were collected from orchards in southern Ontario by J. J. Gill (2000) between 1998 and 2000.



Confluent plate lysates were prepared using the molten agar overlay technique (Adams, 1959). Top agar consisted of 8 g/L nutrient agar (Difco), 2.5 g/L yeast extract (Difco), and 5 g/L food-grade sucrose. Bacteria were suspended to  $1 \times 10^9$  CFU/mL in 0.01 M PB (pH 6.8). One hundred microlitres of the bacterial isolation host suspension was mixed with 100  $\mu$ L of phage suspension in a test tube for 10 min. Three millilitres of molten top agar at 55°C was added, and the mixture was poured over a 90 mm petri plate containing solidified nutrient agar. The plate was swirled to evenly distribute the top agar, and then incubated overnight at 37°C. Following incubation, the plate was flooded with 3 mL of nutrient broth and allowed to sit at room temperature for approximately 15 min. The nutrient broth and top agar were aseptically scraped into a 50 mL round-bottom FEP centrifuge tube and placed on a gyratory shaker for 30 min. Cell debris and top agar were pelleted by centrifuging the mixtures at 8 000  $\times g$  for 25 min. The supernatant was decanted and syringe-filtered as previously described.

#### *Phage Storage Media and Conditions*

Syringe-filtered lysates were stored in nutrient broth at 4°C. Alternatively, the syringe-filtered lysates were centrifuged at 16 000  $\times g$  for 45 min. The supernatant was decanted and discarded. The pellet was resuspended in the medium of choice, usually 0.01 M sodium phosphate buffer (pH 6.8) amended with 100 mM NaCl and 2 mM  $\text{MgCl}_2$  ( $\text{PB}_{\text{salt}}$ ). Suspensions were stored in a sterile tube at 4°C.

#### *Determination of Phage Titre*

Titres of phage stocks were determined using the molten agar overlay method (Adams,

1959). Ten-fold serial dilutions of filtered lysate were prepared in nutrient broth. One hundred microlitres of each dilution was combined with 100  $\mu$ L of exponential phase host culture collected from overnight growth on a plate and suspended in 0.01 M sterile sodium phosphate buffer at pH 6.8 (PB) at  $10^9$  CFU/mL. The mixture of phage and host bacteria were incubated for 10-15 min at room temperature. Three millilitres of molten top agar at 55 °C was added and the mixture of phage, bacteria and top agar was then poured into a petri plate containing nutrient agar. The plate was swirled to evenly distribute the top agar and incubated overnight at 37°C. Following incubation, plaques were counted on any plates where plaques were visible as discrete spots.

Alternatively, the spot lysis technique was used, wherein the agar overlay was seeded with only the bacterial host. Once the poured overlay solidified, 5 or 10  $\mu$ L of each phage dilution was dropped onto the agar overlay and incubated overnight. Spots containing discrete plaques were used to estimate the titre of the original phage suspension.

#### *Revival of the Vineland Phage Collection*

The Vineland phage collection included both liquid lysates and lyophilized lysates that had been prepared according to the ATCC skim milk powder formulation. Lyophilized lysates were rehydrated by the addition of 0.5  $\mu$ L of nutrient broth. Ten microlitres of the mixed material was dropped onto a solidified soft agar lawn seeded with  $1 \times 10^8$  CFU of the isolation host and incubated overnight. Liquid cultures were tested for the presence of viable phage by dropping 10  $\mu$ L of lysate onto a seeded molten agar lawn.

If no plaques developed, the entire volume of lysate was transferred to a 50 mL round-

bottom centrifuge tube and centrifuged at 16 000 xg for 45 min in order to concentrate any viable phages. The supernatant was decanted and the interior surfaces of the centrifuge tube were washed with 1 mL of nutrient broth. The resulting suspension was then tested for the presence of viable phages.

If plaques developed from either the original or the concentrated lysate, a 100-fold dilution series of the lysate was prepared in 0.01 M PB, and plated using the soft agar overlay method. A single, isolated plaque was removed from the top agar using a 1 mL aerosol barrier pipet tip, and placed in 1 mL of nutrient broth. The mixture was vortexed to release phages from the agar matrix. A dilution series was prepared from this sample and the process was repeated for at least two more rounds of single plaque isolation, until a stable plaque morphology was observed. When multiple plaque morphologies were observed, three rounds of single plaque isolation were conducted for each distinct type. If this process resulted in a single, stable plaque morphology, the newly purified lysates were named by adding a letter suffix to their previous designation (ie. phages isolated from the stored  $\phi$ Ea45-1 lysate were named  $\phi$ Ea45-1A and  $\phi$ Ea45-1B).

### *Isolation of Phage DNA*

Two methods were used to isolate phage DNA: organic extraction, and a method using cetyltrimethyl ammonium bromide (CTAB) that was modified from Manfioletti and Schneider (1988). Phages were grown in liquid culture on *E. amylovora* Ea110, regardless of which method was used.

When DNA was isolated using the CTAB method, bacterial nucleic acids were digested

by incubating 100 ng of RNase A and 100 U of DNase I with 10 mL of syringe-filtered phage suspension at room temperature for 15 min. Nucleases were inhibited and phage particles lysed by adding 0.8 mL of 0.5 M EDTA (pH 8.0), 0.5 mg of proteinase K, and incubating the mixture at 45°C for 15 min. 440 µL of 5% CTAB in 0.5 M NaCl was added, and the CTAB:DNA complex was precipitated by cooling the solution on ice for 15 min, followed by centrifugation at 8 000 xg for 10 min. The resulting pellet was resuspended in 1.2 M NaCl. DNA was precipitated by adding 2 mL 95% ethanol, mixing by inversion, and centrifugation at 8 000 xg for 10 min. The DNA pellet was washed with 70% ethanol, allowed to air-dry, and resuspended in 0.5 mL of 10 mM Tris-HCl (pH 8.0), and stored at -20°C.

The organic extraction method was modified from the New England BioLabs lambda DNA isolation protocol<sup>1</sup>. Phage lysates were prepared in liquid culture and syringe-filtered. Ten millilitres of the lysate was concentrated by centrifugation at 16 000 xg for 45 min at 4°C, resuspended in 700 µL SM buffer (50 mM Tris-Cl, pH 7.5; 0.1 M NaCl; 8 mM MgSO<sub>4</sub>), and transferred to a 2 mL microcentrifuge tube. Bacterial nucleic acids were digested by adding 1 µL each of 1 mg/mL DNase I and RNase A and incubating the mixture at 37°C for 30 min. An equal volume of 20% (w/v) PEG 8000 in 2.5 M sodium acetate was added, and the tube was vortexed and incubated on ice for 2 h. The precipitated phages were collected by centrifuging the tubes at 15 000 xg for 10 min at 4°C. The supernatant was aspirated and the phage pellet was resuspended in 500 µL SM buffer. The phage particles were lysed by adding 5 µL of 10% SDS, 500 µL 0.5 M EDTA (pH 8.0), and incubating at 65°C for 15 min. Phage nucleic acids were then purified in a

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<sup>1</sup> Available online: [tools.neb.com/wolbachia/labsite/protocols/lambda\\_phage\\_preps.htm](http://tools.neb.com/wolbachia/labsite/protocols/lambda_phage_preps.htm) [Accessed September 2005]

three-stage organic extraction. An equal volume of buffer-equilibrated phenol was added and mixed gently by inversion for 3 min. Tubes were centrifuged at 13 500 xg for 5 min at room temperature and the upper aqueous phase was removed to a new microcentrifuge tube. The extraction was repeated with an equal volume of 1:1 phenol:chloroform, and then again with an equal volume of chloroform. DNA was precipitated from the aqueous phase of the final extraction by adding sodium acetate to a final concentration of 0.3 M and adding 100% ethanol 100  $\mu$ L at a time, until the phage DNA had just precipitated (about a 1X volume). The precipitated DNA was collected by centrifugation at 15 000 xg for 15 min at 4°C. The supernatant was decanted, the pellet was washed with 70% ethanol and recentrifuged briefly. The ethanol was aspirated and the DNA pellet was allowed to air-dry before being resuspended in 10 mM Tris-HCl (pH 7.5) and stored at -20°C.

#### *Restriction Fragment Length Polymorphisms (RFLPs)*

Restriction endonuclease digestions were conducted using *MvnI* (Roche Diagnostics, Laval, QC), *EcoRI* (Invitrogen Canada, Burlington, ON), *BamHI* (New England Biolabs, Ipswich, MA) and *BglII* (MBI Fermentas, Hanover, MD). *MvnI* digests were conducted at 37°C in a total volume of 25  $\mu$ L. Each reaction contained 1X Buffer M, 5 U *MvnI*, 2-3  $\mu$ g of phage DNA, and sterile distilled water. If the DNA concentration was between 50 ng/ $\mu$ L and 100 ng/ $\mu$ L, the reaction was scaled up to a 50  $\mu$ L volume to accommodate the additional volume of DNA required.

The reaction products were visualized using agarose gel electrophoresis. Reaction products were resolved on a 1% (w/v) agarose gel at 150 V for approximately 75 min. The gel

was stained in a 0.5 µg/mL ethidium bromide solution for 45 min, destained in water for 25 min, and visualized using the GelDoc system (BioRad Laboratories, Hercules, CA).

#### *Endpoint PCR for Group 3 Phages*

Each phage was tested with polymerase chain reaction primers, called PEa1-A and PEa1-B (Schnabel and Jones, 2001), designed to amplify a 304 bp fragment of  $\Phi$ Ea1(h). Amplification reactions were conducted in 25 µL volumes. Each reaction contained 200 µM of each primer, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 mM MgCl<sub>2</sub>, 1.5 U *Taq* (MBI Fermentas), 1X polymerase buffer, and 1.5 µL of phage suspension in nutrient broth. Reactions were run in a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) under the following conditions: 95°C for 2 min; and 30 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s. Reaction products were visualized using agarose gel electrophoresis, as previously described.

#### *Real-time PCR*

TaqMan-style real-time PCR reactions were conducted using two sets of probe and primers, called  $\phi$ -dpo1 and  $\phi$ -dpo2, that were developed by Dr. W. -S. Kim (unpublished) based on the depolymerase (*dpo*) gene of  $\phi$ Ea1 (NCBI Accession no. AJ278614). Detection is based on amplification of a 171 bp ( $\phi$ -dpo1) or 72 bp ( $\phi$ -dpo2) region of the gene. Probes were synthesized by Integrated DNA Technologies (Coralville IA, USA), and were labelled with 6-carboxyfluorescein (FAM) at the 5' end and either Black Hole Quencher 1 (BHQ-1) or Iowa Black (IAbRQ) at the 3' end..

Each reaction was conducted in a total volume of 25 µL, and contained 1X Brilliant

QPCR Master Mix (Stratagene, La Jolla, CA), 200  $\mu$ M of each primer, 100  $\mu$ M probe. Reactions were run in a Stratagene Mx4000 Multiplex Quantitative PCR system (Stratagene) under the following conditions: 95°C for 10 min; 40 cycles of 95°C for 30 s and 60°C for 60 s, with three endpoint fluorescence readings during each amplification segment.

### *Transmission Electron Microscopy (TEM)*

At least 10 mL of each phage suspension was centrifuged at 16 000 xg for 45 min and the resulting pellet was resuspended in 0.5 mL TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0). A drop of the phage suspension was placed on a 400 mesh copper formvar grid. After 1 min the excess sample was drawn off by capillary action using a kimwipe drawn against the edge of the grid. A drop of 2% uranyl acetate was immediately placed on the grid. After 1 min the excess stain was drawn off in the same manner. The sample was allowed to air dry before being examined using a Philips CM10 transmission electron microscope at an accelerating voltage of 80 kV. The microscope was equipped with a digital imaging system from American Microscopy Techniques Corp. Micrographs were taken at the University of Western Ontario, by Ronald Smith. Phage dimensions were automatically calculated from the digital images based on the number of pixels per micron in the field of view at 72,000 or 105,000-fold magnification.

### *Bacterial Isolates*

All bacterial isolates used are shown in Table 2-2. Strains plated from frozen stocks were assessed for uniformity of colony morphology, then subcultured from a single colony for each experiment.

### *Bacterial Culture Media and Conditions*

Bacteria were cultured in 90 mm Petri plates on semi-solid media containing 11.5 g/L nutrient agar (Difco Laboratories, Sparks, MD). *Erwinia* spp. and *Pantoea* spp. were incubated at 28°C for 16-20 h. *E. coli* strains were incubated at 37°C for 16-20 h. Unless otherwise indicated, bacteria were aseptically scraped from the agar surface, suspended in 0.01 M PB pH 6.8, adjusted to  $1 \times 10^9$  CFU/mL ( $OD_{600} = 0.6$ , Beckman DU640 spectrophotometer), and stored on ice until use. The 10X stock of PB (pH 6.8) was prepared as described by Gomori (1955), combining 49 mL of 0.2 M  $Na_2HPO_4$ , 51 mL of 0.2 M  $NaH_2PO_4$ , and 100 mL of distilled water.

Antibiotic production tests were conducted using GA minimal media (Vanneste & Beer, 1992), which contained 20 g/L M D-(+)-glucose (Sigma Chemical Co., St. Louis, MO), 0.3 g/L L-asparagine (Sigma-Aldrich Co., St. Louis, MO), 11.5 g/L  $K_2HPO_4$  (J. T. Baker Chemical Co., Phillipsburg, NJ), 4.5 g/L  $KH_2PO_4$  (Fisher Scientific, Fair Lawn, NJ), 0.12 g/L  $MgSO_4 \cdot 7H_2O$  (J. T. Baker Chemical Co.), and 50 mg/L nicotinic acid (Sigma Chemical Co.). The medium was solidified with Noble Agar (Difco Laboratories, Detroit, MI), 12 g/L for plates, and 7 g/L for overlays. A 2X stock of GA was prepared and sterilized by filtration, and added in a 1:1 ratio to a 2X preparation of melted Noble Agar that had been sterilized by autoclaving.

### *Bacterial Storage Medium and Conditions*

Bacteria were stored at -80°C in a medium containing 4 g/L nutrient broth, 1 g/L yeast extract, 2.5 g/L glucose, 5.7 mM  $K_2HPO_4$ , 1.8 mM  $KH_2PO_4$ , 0.5 mM  $MgSO_4 \cdot 7H_2O$ , and 50% (v/v) glycerol.



Table 2-2. Bacterial isolates used in this study

Isolate	Host Plant	Use	Reference or Source
<i>Erwinia amylovora</i>			
Ea6-4	<i>Pyrus communis</i>	Phage Growth; Host Range; Blossom assays	(Jeng et al., 2001)
Ea17-1-1	<i>Pyrus communis</i>	Phage Growth; Host Range	(Jeng et al., 2001)
Ea29-7	<i>Malus X domestica</i>	Phage Growth; Host Range	(Gill et al., 2003)
Ea110	<i>Malus X domestica</i>	Phage Growth; Host Range	(Ritchie & Klos, 1977)
EaD-7	<i>Pyrus communis</i>	Phage Growth; Host Range	(Jeng et al., 2001)
EaG-5	<i>Pyrus communis</i>	Phage Growth; Host Range	(Jeng et al., 2001)
Ea273	<i>Malus X domestica</i>	Antibiotic testing	Beer, S.V. <sup>a</sup>
Ea1/79	<i>Cotoneaster sp.</i>	Host Range	(Falkenstein et al., 1988)
<i>Pantoea agglomerans</i>			
Eh252	<i>Malus X domestica</i>	Antibiotic testing	(Vanneste, Yu, & Beer, 1992)
C9-1	<i>Malus X domestica</i>	Antibiotic testing	(Ishimaru, Klos, & Brubaker, 1988)
E325	<i>Malus X domestica</i>	Host Range	(Pusey, 1997)
Eh21-5	<i>Pyrus communis</i>	Phase 2 carrier candidate testing	A. M. Svircev <sup>b</sup> (1998)
1-28b	<i>Malus X domestica</i>	Phase 2 carrier candidate testing	A. M. Svircev (1997)
13P-4 (2) II	<i>Pyrus communis</i>	Phase 2 carrier candidate testing	A. M. Svircev
13P-5 (1) II	<i>Pyrus communis</i>	Phase 2 carrier candidate testing	A. M. Svircev
17P-3 (2) II	<i>Pyrus communis</i>	Phase 2 carrier candidate testing	A. M. Svircev (1997)
21-1-1-2	<i>Pyrus communis</i>	Phase 2 carrier candidate testing	A. M. Svircev (1998)
21-1-5-1	<i>Pyrus communis</i>	Phase 2 carrier candidate testing	A. M. Svircev (1998)
39C II	<i>S. aucuparia</i> (European Mountain Ash)	Phase 2 carrier candidate testing	A. M. Svircev (Royal Botanical Gardens, Burlington, ON)

Strain	Host Plant	Use	Reference or Source
39L II 2	Dorothea Crab	Phase 2 carrier candidate testing	A. M. Svircev (1998, Royal Botanical Gardens, Burlington, ON)
39U II	<i>Cotoneaster</i> spp.	Phase 2 carrier candidate testing	A. M. Svircev (1998, Royal Botanical Gardens, Burlington, ON)
39V II	<i>Cotoneaster amigus</i>	Phase 2 carrier candidate testing	A. M. Svircev (1998, Royal Botanical Gardens, Burlington, ON)
39W II	<i>Cotoneaster</i> (Lucida)	Phase 2 carrier candidate testing	A. M. Svircev (1998, Royal Botanical Gardens, Burlington, ON)
Other			
244 isolates collected from blossoms in southern Ontario	generally, <i>Pyrus communis</i> or <i>Malus X domestica</i>	Phase 1 carrier candidate testing	A. M. Svircev; 1997 and 1998
<i>Pectobacterium carotovora</i> , Ecc26			D. Cuppels <sup>c</sup>
<i>Pseudomonas fluorescens</i> A506		field testing	(Lindemann & Suslow, 1987)
<i>Pseudomonas syringae morsprunorum</i> , psm7		Environmental Impact Testing	Teresa Ainsworth <sup>b</sup>
<i>P. syringae morsprunorum</i> , psm61		Environmental Impact Testing	Teresa Ainsworth
<i>P. syringae morsprunorum</i> , psm37		Environmental Impact Testing	Teresa Ainsworth
<i>P. syringae papulans</i> , psp mutsu	<i>Malus X domestica</i> , mutsu	Environmental Impact Testing	D. Hunter <sup>b</sup>
<i>Xanthomonas campestris</i> pv. <i>pruni</i> , Xc69	<i>Prunus</i> sp.	Environmental Impact Testing	Teresa Ainsworth
<i>Xanthomonas campestris</i> pv. <i>pruni</i> , Afl	<i>Prunus</i> sp.	Environmental Impact Testing	Teresa Ainsworth

<sup>a</sup> CUCPB, Cornell University Collection of Phytopathogenic Bacteria

<sup>b</sup> Agriculture & Agri-Food Canada, Southern Crop Protection and Food Research Centre, Vineland, ON

<sup>c</sup> Agriculture & Agri-Food Canada, Southern Crop Protection and Food Research Centre, London, ON

### *Antibiotic Production by P. agglomerans*

*P. agglomerans* Eh21-5 was harvested from a fresh, overnight plate culture and washed once with GA medium to remove trace nutrients from the rich medium. Cells were suspended in 1 mL 0.01 M PB, centrifuged at 13 000 xg for 5 min, and resuspended in 1 mL of fresh PB. A 125 mL Erlenmeyer flask containing 25 mL of liquid GA medium was inoculated with the resuspended cells. *P. agglomerans* C9-1 and *E. amylovora* Ea110 were used as positive and negative controls, respectively.

After 48 h, cells were removed from the culture media by centrifuging the suspension at 8 000 xg for 25 min and syringe-filtering the supernatant through a surfactant-free cellulose acetate filter with 0.2 µm-diameter pores. Filter-paper discs, 6 mm in diameter, were soaked in the filtered supernatant for 2-3 min. Excess liquid was blotted from the disc, and it was placed on a GA plate that had been overlaid with GA top agar seeded with  $1 \times 10^9$  CFU/mL of the indicator strain. Alternatively, 30 µL of filtered growth media was placed on the disc and allowed to soak through it. Plates were incubated at 28°C, and checked after 24 and 48 h. A zone of inhibited growth on the bacterial lawn around the location of the infiltrated disc indicated production of an antibiotic compound by the test strain.

### *Amplification of a Gene from the Pantocin A Biosynthetic Cluster*

*P. agglomerans* carrier candidates were tested for the presence of the *paaB* gene using primers designed to amplify an 813 bp developed by Jin et al (2003). Reactions were conducted in 50 µL volumes. Each reaction contained 200 µM of each primer, 1.5 mM MgCl<sub>2</sub>, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1 U of *Taq* polymerase (MBI Fermentas), 1X

ThermoPol buffer, and 5  $\mu$ L of template. Reactions were carried out in a GeneAmp 9700 thermal cycler (Applied Biosystems) under the following conditions: 95°C for 2 min; 30 cycles of 95°C for 15 s, 53°C for 15 s, and 72°C for 30 s. Reaction products were visualized on a 0.8% agarose gel, as previously described.

Templates were prepared by scraping a small amount of bacterial cells from a nutrient agar plate and suspending them in 0.5 mL of sterile, distilled water. *P. agglomerans* Eh252 and C9-1 were used as positive controls. *E. amylovora* Ea6-4 and Ea273, *E. pyrifoliae* 1/96, *P. fluorescens* A506, water, and master mix were used as negative controls.

#### *Host Range of the Vineland Phage Collection on E. amylovora and P. agglomerans Isolates*

Bacterial isolates were tested for *in vitro* susceptibility to phage infection using the spot method, a modification of the soft agar overlay method. A bacterial lawn was created by seeding 3 mL of molten top agar with 100  $\mu$ L of  $1 \times 10^9$  CFU/mL of the test isolate and pouring the mixture over a nutrient agar plate. Ten microliters of a  $1 \times 10^7$  PFU/mL phage suspension were dropped onto the overlay and the plate was incubated overnight.

Two hundred and fifty-six bacterial isolates that had previously been collected from the aerial tissue of rosaceous hosts in southern Ontario were considered as part of the carrier selection process. Ninety-eight of these had previously been identified as *Pantoea agglomerans* based on PCR amplification of a 16S-23S intergenic region, as described by Jeng et al (2001) (A.M. Svircev, unpublished data).

The host range of the Vineland phage collection was also tested on the six *E. amylovora* isolation hosts, several strains of *P. agglomerans* from other sources, and on bacterial strains

isolated from local orchards: *Pseudomonas syringae morsprunorum* Psm7, *P. syringae morsprunorum* Psm61, *P. syringae morsprunorum* Psm37, *P. syringae papulans* psp mutsu, *Xanthomonas campestris* pv. *pruni* Xc69, and *X. campestris* pv. *pruni* Afl.

### *Efficacy of Carrier Candidates and Phages in Blossom Assays*

The biocontrol activity of individual bacteriophages, carrier bacteria, and combinations of the two was evaluated using a pear blossom bioassay. Pear shoots bearing dormant buds were harvested in later winter. Budwood was bundled, loosely wrapped in clear plastic, and stored at 1°C until use. The lower 15 to 20 cm of each branch was surface disinfected by dipping them in 70% ethanol and then cutting 2 to 5 cm off of the bottom with clean pruning shears. Budwood was then forced to form blossoms by placing them in tap water at 20°C. Branches were surface disinfected, re-cut, and placed into fresh water every 3 to 4 d.

Individual newly opened blossoms were collected by hand and placed into sterilized glass scintillation vials containing sterile tap water, such that the peduncle extended through a hole drilled in the lid of each vial.

To determine the minimum concentration of *E. amylovora* Ea6-4 needed to produce full disease in the untreated control, 10 µL of *E. amylovora* Ea6-4 at 1x10<sup>8</sup> CFU/mL or 1x10<sup>6</sup> CFU/mL or 1x10<sup>4</sup> CFU/mL was applied to each of five blossoms. PB was used as a control. This entire experiment was repeated at a later date with the following changes: sets of 10 blossoms were used for each treatment; treatments were repeated in two more sets of 10 blossoms, using independently prepared bacterial cultures for each set and suspensions of *E. amylovora* Ea6-4 prepared to 1x10<sup>8</sup> CFU/mL, 1x10<sup>7</sup> CFU/mL, 1x10<sup>6</sup> CFU/mL, 1x10<sup>5</sup> CFU/mL, and PB.

To screen for protective effects of previous treatment with a phage or candidate carrier bacterium, 10 individual blossoms were treated with 10  $\mu\text{L}$  of phage at  $1 \times 10^8$  PFU/mL or with 10  $\mu\text{L}$  of carrier bacteria at  $1 \times 10^8$  CFU/mL, and then challenged by applying 10  $\mu\text{L}$  of *E. amylovora* Ea6-4 at  $1 \times 10^8$  CFU/mL. Controls consisted of treatment with 10  $\mu\text{L}$  of PB followed by 10  $\mu\text{L}$  of *E. amylovora* Ea6-4, or 20  $\mu\text{L}$  of PB with no subsequent application of *E. amylovora*. This inoculation procedure was repeated using independently prepared bacterial cultures. Depending on the number of phages or bacteria being screened, the interval between the treatment and pathogen applications was approximately 10 to 20 min.

Vials were held in plastic racks, and loosely sealed inside large plastic bins that had been flooded with 500 mL of tap water in order to maintain a high relative humidity. Blossoms were incubated at room temperature, about 22 to 25°C. After 4 d, disease symptoms were evaluated according to the rating scale in Figure 2-2.

Results were analyzed in SAS (Statistical Analysis Systems 8.2; SAS Institute, Cary, NC) using the general linear model (PROC GLM). Disease severity index ratings from the competition assay were converted to a percent scale based on the total hypanthium and ovary surface area showing visible necrosis (0 = 0%, 1 = 20%, 1.5 = 38%, 2 = 48%, 2.5 = 55%, 3 = 61%, 3.5 = 68%, 4 = 82%, 5 = 100%) . The differences among different levels of pathogen inoculum were determined using Duncan's multiple range test. The differences in disease severity between blossoms treated with buffer and blossoms treated with an individual phage or carrier candidate were analyzed using a one-sided Dunnett's test for multiple comparisons to the specified PB control.

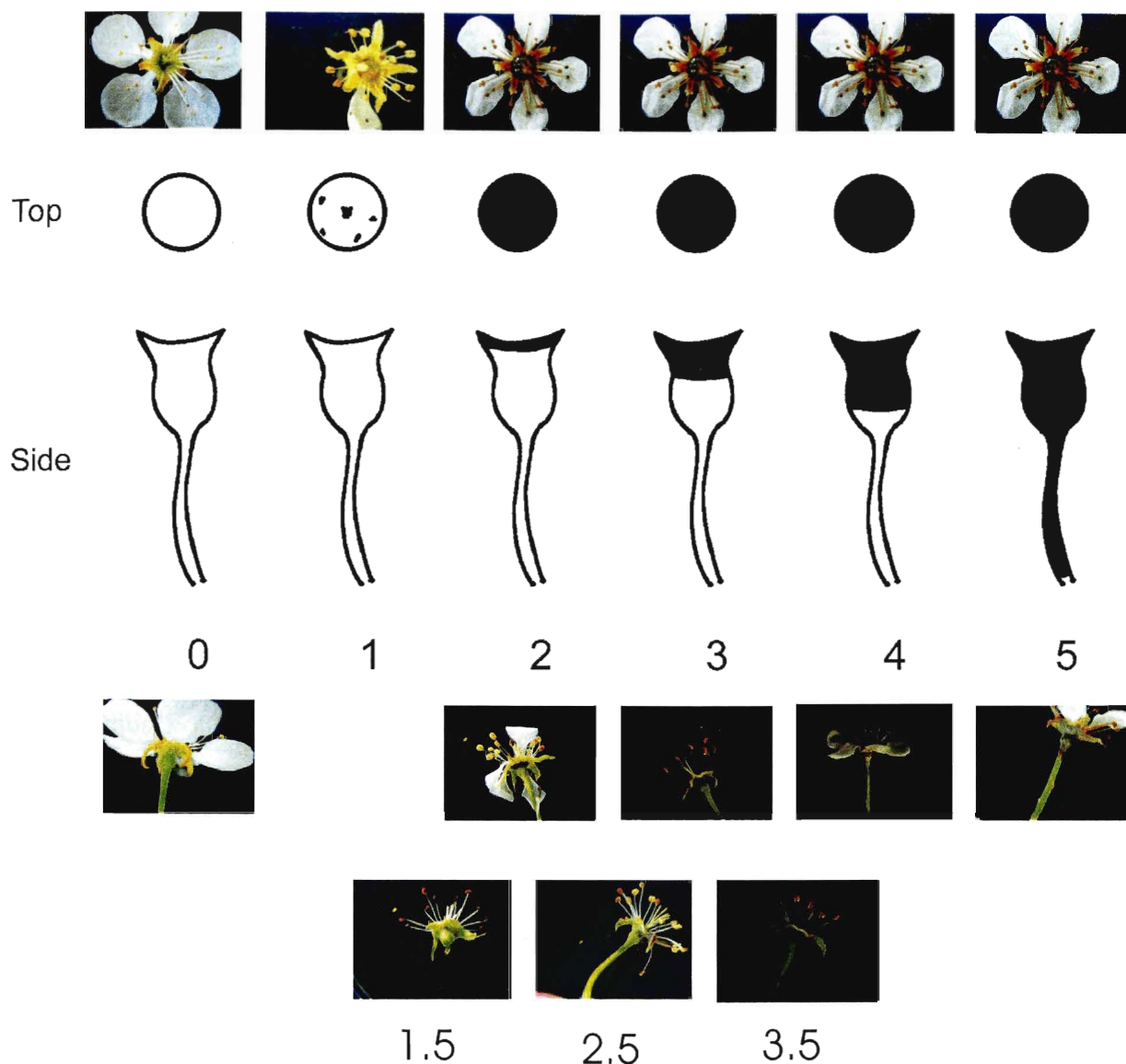


Figure 2-2. Rating scale describing the severity of fire blight symptoms in the pear blossom bioassay. Integer values are based on the vertical progression of necrosis through the blossom head to the peduncle. Half-scale ratings are based on the furthest vertical progression of symptoms, less a half point if symptom progression extends less than half the way around the nectary at that distance. [Black and white graphic is taken from Gill (2000); Blossom photos were taken by S. M. Lehman]

## Results

### *Revival of the Vineland Phage Collection*

When this work commenced, three stored copies of the Vineland collection existed: filtered liquid lysates prepared by J. J. Gill ca. 2000, stored at 4°C in nutrient broth over a drop of chloroform (Gill, 2000); filtered lysates prepared by M. Schmuck ca. 2003 from the Gill lysates, stored at 4°C in nutrient broth; and lyophilized lysates prepared by J. J. Gill ca. 2000 in skim-milk medium. No viable phages were recovered from the lyophilized lysate. All of the phages collected by J. J. Gill (Gill, 2000), except for  $\phi$ Ea31-1,  $\phi$ Ea45-2 and  $\phi$ Ea50-2, were recovered from at least one of the liquid lysates.

A stable plaque morphology was observed for most phage isolates. However, certain isolates, most notably the group 1 phages, have a variable plaque morphology. Successive rounds of single plaque isolation, in which the different morphologies were carefully noted, always yielded the same mixture of plaque appearances. Generally, plaques noted to be of one appearance would resemble the other type if the plate was allowed to incubate longer. When multiple plaque morphologies were observed, comparative RFLP analysis was conducted on the genomic DNA of each isolate. If the same RFLP pattern was observed for each one, they were assumed to be the same phage and the letter suffixes were dropped.

### *Molecular Characterization of Phage Collection*

RFLPs were used to compare the recovered phages to the original collection, as described by Gill (2000). In that work, *Bam*HI, *Eco*RI, *Bgl*II, and *Tha*I were used to digest DNA. Only *Tha*I digested all phage genomes, and gave a unique pattern for all the described groupings. Therefore



*MvnI*, which is an isoschizomer of *ThaI*, was used in the present study. The phages were also classified based on two PCR assays. The endpoint PCR assay was taken from Schnabel and Jones (2001), who cloned and sequenced a 1.8 kB *BglIII* fragment of  $\phi$ Ea1, and designed primers to amplify a 304 bp region of it. The real-time PCR assays use two primer and probe sets designed by Dr. W. -S. Kim, based on the sequence of the  $\phi$ Ea1 depolymerase gene. Table 2-3 shows the results of these assays.

### *Transmission Electron Microscopy*

Some of the phages selected for phage-carrier biopesticide development were examined by TEM. The sizes and family assignments of these phages are described in Table 2-4. Phages with short tails are members of the *Podoviridae* (Figure 2-3B). Of the remaining, long-tailed, phages, some were observed in both the contracted and uncontracted state, and have therefore been classified as *Myoviridae* (Figure 2-3 ACD). Curved tails with no apparent narrowing of the neck (the proximal end of the tail), such as those observed in the preparations of  $\phi$ Ea35-4 and  $\phi$ Ea35-5, are characteristic of the non-contractile *Siphoviridae* (Figure 2-3E).

The morphologies of  $\phi$ Ea9-5,  $\phi$ Ea 21-3, and  $\phi$ Ea31-3 that were determined in this study are consistent with those reported by Gill et al (2003). The morphologies of the remaining phages were not previously reported. However, the morphology of  $\phi$ Ea35-4 is consistent with the morphologies reported for other group 2 phages (Gill, 2000; Gill et al., 2003), while the morphology of  $\phi$ Ea35-5 is not the *Podoviridae* morphology that was reported for other group 4 phages (Gill, 2000; Gill et al., 2003).

Table 2-3. Molecular characterization of the revived phage collection. RFLP confirmation of the group assignment made by Gill (2000) is given, as are the results of three PCR assays.

Gill Group <sup>a</sup>	Phage Name	RFLP <sup>b</sup> Confirmation	PCR Assay <sup>c</sup>			Isolation Host
			$\phi$ Ea1	$\phi$ -dpo1	$\phi$ -dpo2	
1	$\phi$ Ea10-2		-	-		Ea6-4
	$\phi$ Ea10-3		+	+		EaD-7
	$\phi$ Ea10-4	1	-	(+)		EaG-5
	$\phi$ Ea21-1		+	+		Ea110
	$\phi$ Ea21-2	1	-	-		EaG-5
	$\phi$ Ea21-3	1	+	+	(+)	EaD-7
	$\phi$ Ea21-4	1	-	(+)		Ea6-4
	$\phi$ Ea31-1		+	+		Ea110
	$\phi$ Ea35-2		+	+		Ea17-1-1
2	$\phi$ Ea10-5		+	+		Ea110
	$\phi$ Ea10-6		-	+		Ea29-7
	$\phi$ Ea31-2		-	(+)		EaD-7
	$\phi$ Ea31-4		+	+		Ea29-7
	$\phi$ Ea35-4	2	-	(+)	+	Ea110
	$\phi$ Ea35-6		+	+		EaD-7
	$\phi$ Ea35-7A		-	(+)		Ea29-7
	$\phi$ Ea35-7B		-	-		Ea29-7
	$\phi$ Ea35-7C		-	-		Ea29-7
3a	$\phi$ Ea10-7	3a	+	+		Ea29-7
	$\phi$ Ea10-8		+	+		Ea29-7
	$\phi$ Ea10-9	3a	+	+		Ea110
	$\phi$ Ea10-10		+	+		Ea110
	$\phi$ Ea10-11		+	+		Ea17-1-1
	$\phi$ Ea10-13	3a	+	+		Ea6-4
	$\phi$ Ea10-14		+	+		Ea110
	$\phi$ Ea10-15	3a	+	+		Ea110
	$\phi$ Ea31-3	3a	+	+	+	Ea29-7
3b	$\phi$ Ea46-2		+	+		EaD-7
3c	$\phi$ Ea1	3c	+	+		Ea110
4	$\phi$ Ea10-12		-	(+)		EaG-5
	$\phi$ Ea10-16		-	(+)		Ea17-1-1
	$\phi$ Ea35-3		+	+		Ea110
	$\phi$ Ea35-5	4	+	+		EaD-7

Gill Group <sup>a</sup>	Phage Name	RFLP <sup>b</sup> Confirmation	PCR Assay <sup>c</sup>			Isolation Host
			PEa1	φ-dpo1	φ-dpo2	
5	φEa9-2		-	-		Ea17-1-1
	φEa9-4		-	+		EaG-5
	φEa9-5	5	+	+	(+)	Ea6-4
6	φEa51-1	6	+	+	-	Ea17-1-1
	φEa51-2	3a	-	(+)		Ea110
	φEa51-3		-	-		Ea6-4
	φEa51-4	6	-	(+)		Ea29-7
	φEa51-6	6	-	(+)		Ea6-4
	φEa51-7	6	-	(+)		Ea29-7
	φEa51-8A		-	-		Ea17-1-1
	φEa51-8B		-	-		Ea17-1-1
	φEa51-8C		-	-		Ea17-1-1
Ungrouped	φEa9-3		+	+		Ea29-7
	φEa10-1	1	-	-	(+)	Ea17-1-1
	φEa45-1A	ungrouped	-	(+)		Ea29-7
	φEa45-1B		-	+	+	NA
	φEa45-3		+	+		EaG-5
	φEa46-1A1		+	+		EaD-7
	φEa46-1A2	Not digested	+	+	(+)	EaD-7
	φEa46-1A3		+	+		EaD-7
	φEa46-1C		+	+		EaD-7
	φEa46-1E		+	+		EaD-7
	φEa50-3		+	(+)		Ea17-1-1
	φEa10-17	3b		(+)		Ea29-7

<sup>a</sup> The RFLP groupings assigned by Gill (2000), based on *Tha*I, *Bgl*II, *Bam*HI, *Eco*RI.

<sup>b</sup> RFLPs are based on *Mvn*I (an isoschizomer of *Tha*I), *Bgl*II, *Bam*HI, *Eco*RI.

<sup>c</sup> Amplification results from stocks containing at least 1x10<sup>6</sup> PFU/mL are shown as: +, positive; (+) weak positive; -, negative. A blank cell indicates that the phage was not tested in that assay.

Table 2-4. Family-level characterization of selected *E. amylovora* phages based on TEM.

Phage	Family	Approximate Head Width (nm)
φEa9-5	<i>Podoviridae</i>	59-65
φEa10-1	<i>Myoviridae</i>	70-81
φEa21-3	<i>Myoviridae</i>	60-80
φEa31-3	<i>Podoviridae</i>	58-66
φEa35-4	<i>Siphoviridae</i>	113-129
φEa35-5	<i>Siphoviridae</i>	53-61
φEa45-1B	<i>Myoviridae</i>	60
φEa46-1A2	<i>Myoviridae</i>	105-113
φEa51-1	<i>Myoviridae</i>	113

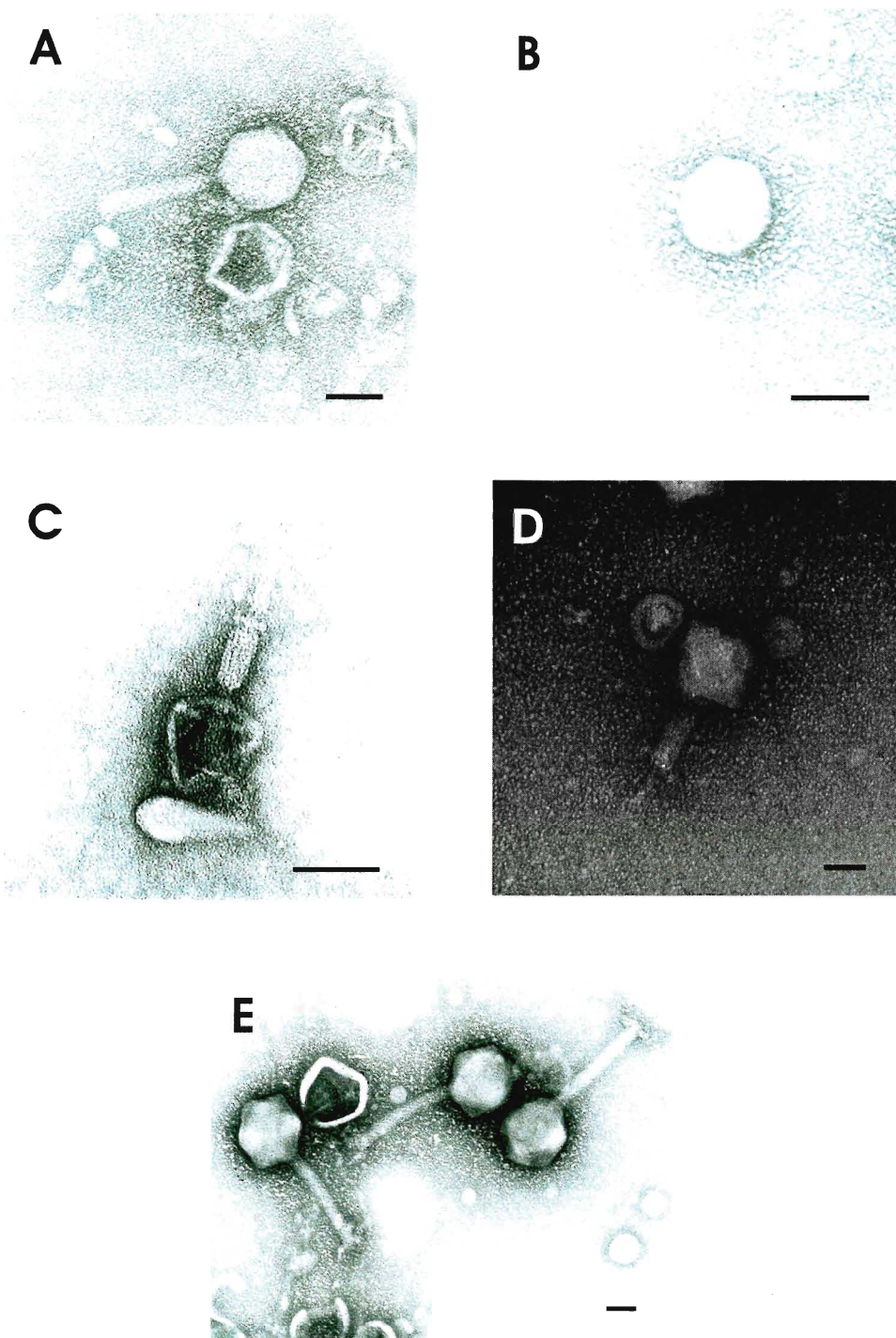


Figure 2-3. TEM of five phages of *E. amylovora*. A)  $\Phi$ Ea10-1, B)  $\Phi$ Ea31-3, C)  $\Phi$ Ea45-1B, D)  $\Phi$ Ea46-1A2, E)  $\Phi$ Ea35-4. [micron marker = 50 nm]

### *Antibiotic Production by P. agglomerans*

Several of the candidate carrier bacteria were tested for antibiotic production. Table 2-5 summarizes the results of those tests. The weaker zones of inhibition on *E. amylovora* Ea6-4 and Ea110 were produced by filter discs that had been inoculated with 30  $\mu$ L of the respective aseptic growth medium, instead of having been soaked in it. The antibiotic activity of media in which *P. agglomerans* Eh21-5 and C9-1 had been grown was still present at a similar level after one week of storage at 4°C.

Figure 2-4 shows the zones of inhibition produced by different antibiotics. The clear, inner zone of inhibition produced by 39CII and C9-1 is similar to that reported for pantocin A (Ishimaru et al., 1988; Vanneste et al., 1990). The larger, more diffuse zone of inhibition produced by C9-1 pantocin B appears similar to the 12 mm zones of inhibition produced by Eh21-5 and many of the other orchard *P. agglomerans* isolates. The clear zone of inhibition produced by 13P-5(2)II is different from either pantocin A or B.

The *paaB* primers amplified a DNA fragment just larger than 750 bp from Eh252, PaC9-1, 39CII, and 1-28b (data not shown).

Table 2-5. Growth inhibition of *Erwinia* spp. by *P. agglomerans* antibiotics. The radii of growth inhibition (mm) is given for each producer-indicator combination.

Producer		Indicator			
		<i>E. amylovora</i> Ea273	<i>E. amylovora</i> Ea6-4	<i>E. amylovora</i> Ea110	<i>E. pyrifoliae</i> Ep 1/96
<i>P. agglomerans</i>	C9-1	3; (12) <sup>a</sup>	5	3; (18)	- <sup>b</sup>
	Eh252	3	NT <sup>c</sup>	NT	NT
	Eh21-5	(12)	(12)	(12)	-
	39CII	3	4	3	NT
	39LII(2)	(12)	(12)	(12)	NT
	39UII	(12)	(12)	(12)	NT
	39WII	(12)	(12)	(12)	NT
	13P-4(1)II	(12)	(12)	(12)	NT
	13P-5(2)II	8	13	11	NT
	21-1-1-2	(12)	(12)	(12)	NT
	21-1-5-1	-	-	-	NT
	1-28b	(12)	(12)	(12)	NT
<i>E. amylovora</i>	Ea273	-	-	-	NT
uninoculated GA media		-	-	-	-

<sup>a</sup> numbers in brackets indicate weak inhibition in a zone of the given radius.

<sup>b</sup> “-” indicates that no growth inhibition was observed

<sup>c</sup> “NT” indicates that the combination was not tested.

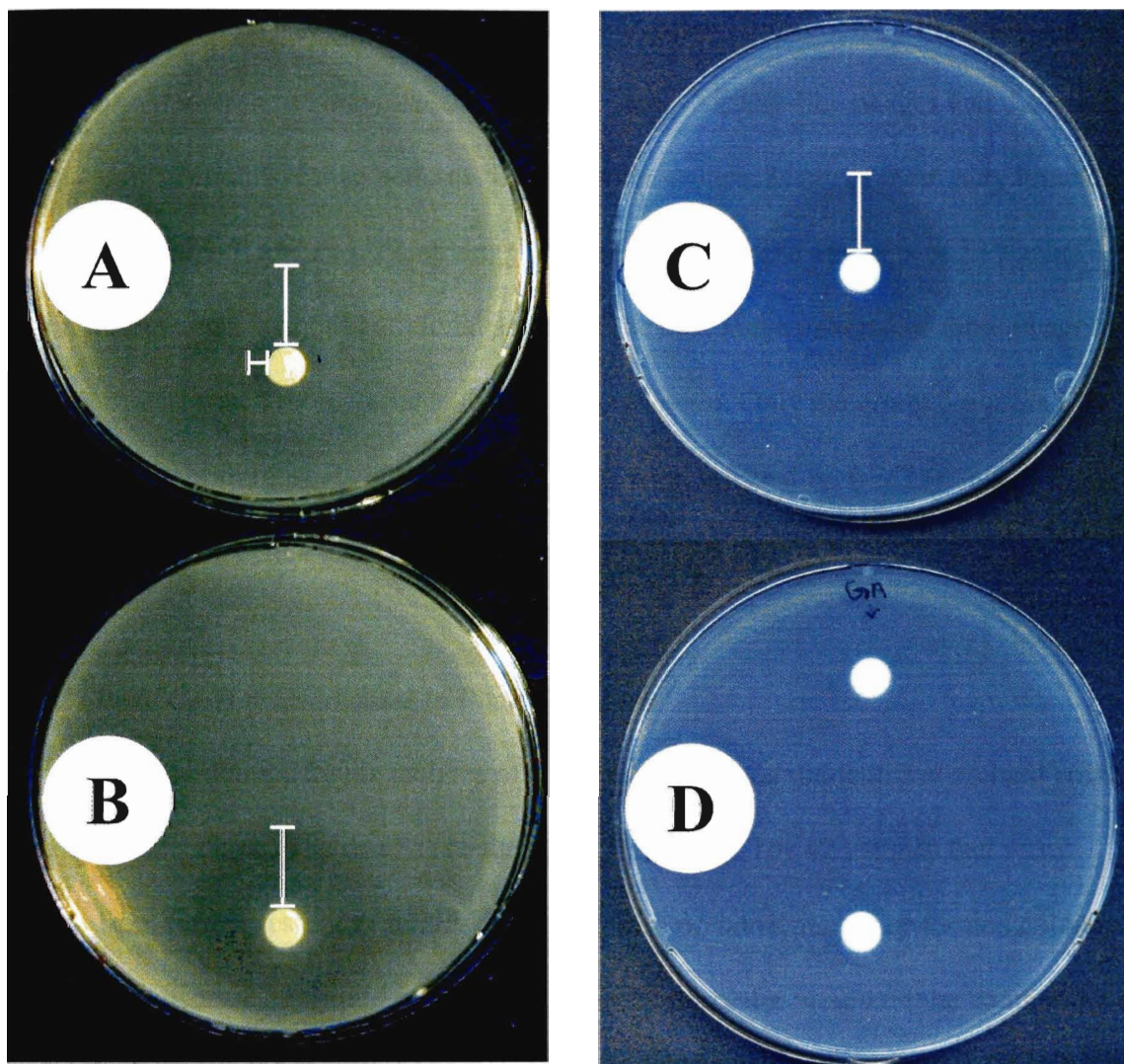


Figure 2-4. Growth inhibition of *E. amylovora* by *P. agglomerans* antibiotics. Hatched lines indicate the dimensions of the zones of inhibition produced by extracellular metabolites of A) strain C9-1, B) strain Eh21-5, C) strain 13P-5(2)II, and D) negative controls: uninoculated GA (top) media and *E. amylovora* Ea273 (bottom).



### *Host Range of the Vineland Phage Collection*

Table 2-6 summarizes the host ranges of the Vineland collection on *E. amylovora* and *P. agglomerans*. Among the *E. amylovora* strains, the group 3  $\phi$ Ea1-like phages and the group 6 phages have different host range patterns than the other groups. Many of them only infected Ea110, EaD-7, Ea29-7, and Ea1/79, strains that produce noticeable amounts of EPS even when grown on nutrient agar without additional sucrose. The host range patterns of these phages in *P. agglomerans* were much more variable than in *E. amylovora*. Only the group 3 phages showed noticeable trends, with most *P. agglomerans* strains being infected by  $\phi$ Ea1 (group 3C), and few strains being infected by the local group 3 isolates.

### *Susceptibility of Carrier Candidates to Phage Infection*

Two hundred and fifty-six isolates of *P. agglomerans* and unidentified orchard epiphytic bacteria were considered. The results are shown in Figure 2-5. The 42 isolates that were eliminated from further testing generally did not flourish on nutrient agar or formed colonies that were not easily dispersed in liquid media. Therefore these isolates would not be compatible with the methods currently employed for large-scale biopesticide production.

In Phase 1 of the screening process, the remaining 214 isolates were tested for susceptibility to infection by 10 of the Vineland *Erwinia* phages:  $\phi$ Ea9-4,  $\phi$ Ea9-5,  $\phi$ Ea10-1,  $\phi$ Ea21-3,  $\phi$ Ea31-3,  $\phi$ Ea35-4,  $\phi$ Ea35-5,  $\phi$ Ea45-1B,  $\phi$ Ea46-1A2,  $\phi$ Ea51-1. These phages had been chosen based on their performance in the *in planta* pear blossom assay, and for their diversity in terms of original isolation site and original RFLP grouping (Gill, 2000).

Of the 109 isolates that were infected by 5 to 10 phages, 12 were selected for the phase 2

Table 2-6. Host range of the Vineland phage collection on *Erwinia* and *Pantoea*. For each combination of phage group and host strain is indicated the number of phages in that group that “+” infected the host, “(+)” weakly infected the host, or “-” did not infect the host.

Host	Group 1 <sup>a</sup>			Group 2			Group 3A			Group 3B			Group 3C		
	+	(+)	-	+	(+)	-	+	(+)	-	+	(+)	-	+	(+)	-
<i>E. amylovora</i>															
Ea6-4	7	0	0	6	1	3	2	2	4	0	0	1	0	0	1
Ea17-1-1	7	0	0	4	1	5	2	0	6	0	0	1	0	0	1
Ea110	7	0	0	10	0	0	8	0	0	1	0	0	1	0	0
EaD-7	7	0	0	9	1	0	8	0	0	1	0	0	1	0	0
Ea29-7	7	0	0	7	3	0	8	0	0	1	0	0	1	0	0
EaG-5	7	0	0	2	3	5	2	0	6	0	0	1	0	0	1
Ea1/79	7	0	0	10	0	0	8	0	0	1	0	0	1	0	0
<i>P. agglomerans</i>															
E325	0	5	2	3	5	2	0	1	7	0	1	0	0	0	1
1-28b	5	0	2	7	0	3	2	1	5	0	0	1	1	0	0
13P-5(2)II	4	1	2	7	0	3	2	1	5	0	0	1	1	0	0
Eh21-5	4	0	3	4	1	5	0	3	5	0	0	1	1	0	0
39CII	0	5	1	3	1	6	0	0	8	0	0	1	1	0	0
39LII	5	1	0	3	4	3	1	1	6	0	1	0	1	0	0

Host	Group 4			Group5			Group 6			Ungrouped		
	+	(+)	-	+	(+)	-	+	(+)	-	+	(+)	-
<i>E. amylovora</i>												
Ea6-4	3	0	1	2	1	0	1	0	5	11	0	6
Ea17-1-1	3	0	1	3	0	0	1	1	3	11	0	6
Ea110	4	0	0	3	0	0	5	0	0	15	2	0
EaD-7	3	1	0	0	3	0	5	0	0	17	0	0
Ea29-7	3	1	0	0	3	0	5	0	0	16	1	0
EaG-5	3	0	1	3	0	0	1	0	4	9	2	6
Ea1/79	4	0	0	3	0	0	5	0	1	17	0	0
<i>P. agglomerans</i>												
Pa E325	0	0	4	0	0	3	4	0	1	0	4	13
1-28b	2	1	0	2	0	1	0	0	6	5	1	11
13P-5(2)II	1	0	3	0	0	3	1	1	3	5	3	9
Eh21-5	1	3	0	1	1	0	3	0	2	6	4	7
39CII	1	1	1	2	0	1	0	1	4	7	3	6
39LII	2	0	1	3	0	0	1	3	1	7	4	5

<sup>a</sup> RFLP group assignments are those of Gill (2000).

screening. They were selected to represent a range of geographical locations and host plant species, as well as varied performance in Phase 1 to compensate for the possibility that the 10 phages used in that screen were less diverse, and therefore a more biased selection tool, than originally thought. Table 2-7 shows the results of the phase 2 screening. Four carrier candidates, 21-5, 39LII2, 39VII, and 39WII, were infected by more than 30 of the 54 phages.

### *Susceptibility of Common Orchard Bacteria to Infection by E. amylovora Phages*

Gram-negative bacteria that had previously been isolated from southern Ontario orchards were screened for susceptibility to *E. amylovora* phages in order to assess the potential impact of a phage-based biopesticide on orchard microbial ecology. None of the *Pseudomonas syringae* pv. *morsprunorum* isolates or *Xanthomonas campestris pruni* isolates were infected by  $\phi$ Ea1 or any of the *E. amylovora* phages in the Vineland collection. Some of the group 1, 2, 3a, and ungrouped phages infected *P. syringae* pv. *papulans*, Psp mutsu, weakly for the most part, and in a pattern similar to that on *P. agglomerans* E325.

### *Efficacy of Carrier Candidates and Phages in Blossom Assays*

In the first set of experiments designed to optimize the pathogen inoculum, a  $1 \times 10^8$  CFU/mL, suspension of *E. amylovora* Ea6-4 was required in order to cause an average symptom severity of 80% or greater ( $P < 0.05$ ). In the more detailed experiment conducted later, treatment with  $1 \times 10^6$  CFU/mL was the minimum inoculum needed. Treatment with  $1 \times 10^5$  CFU/mL of *E. amylovora* caused a mean of 55% disease, which was greater than the uninoculated blossoms,

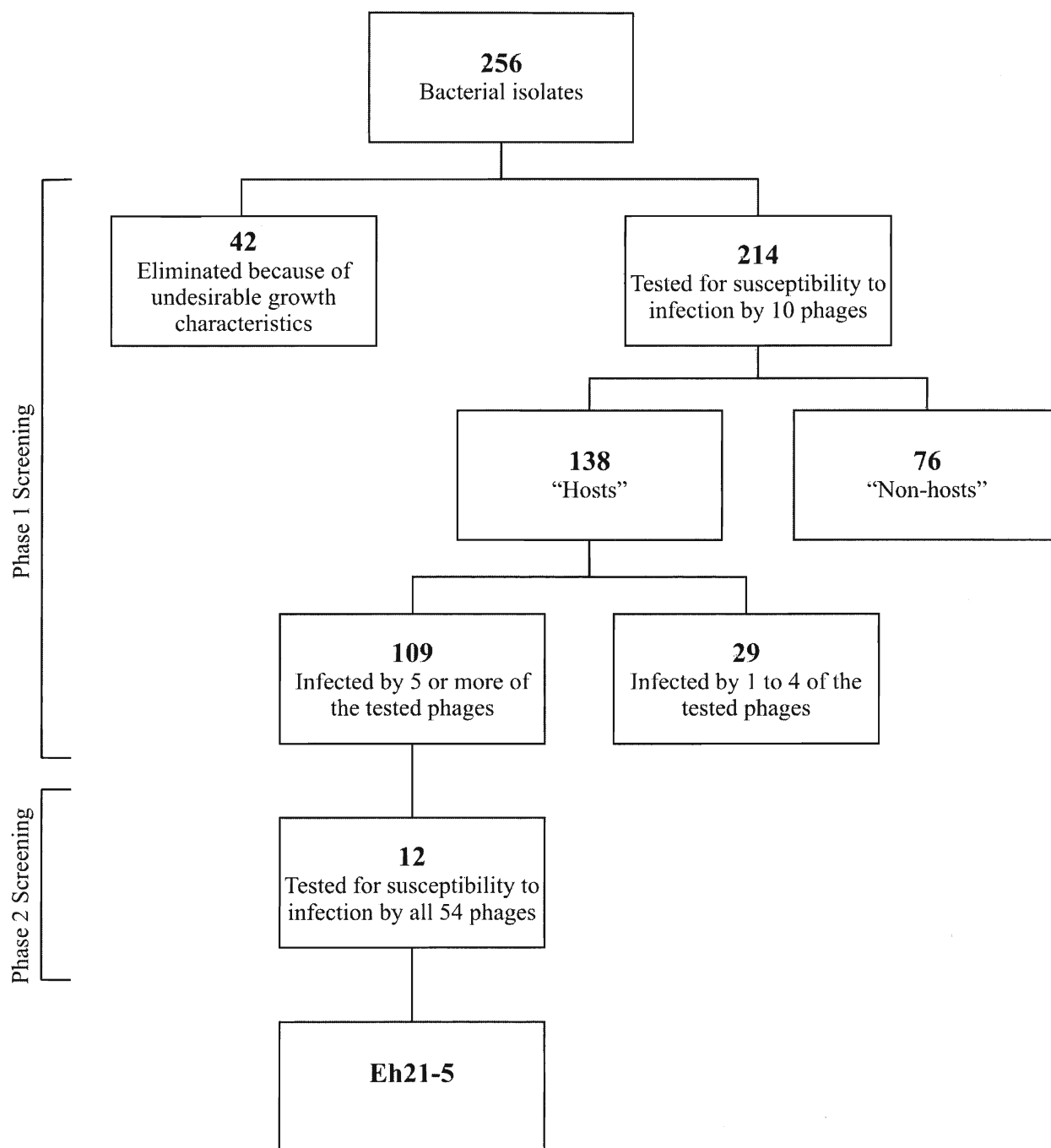


Figure 2-5. Screening bacterial epiphyte isolates for susceptibility to infection by *E. amylovora* phages.

Table 2-7. Host range of *Erwinia* phages on *P. agglomerans* carrier candidates. Phage groupings are those assigned by J. J. Gill (2000), including the ungrouped (“NA”) phages. Shaded cells indicate that the bacterial isolate was susceptible to infection by the phage. White cells indicate that the isolate was not susceptible. “NT” indicates that the combination was not tested.

Group	Phage	Orchard Epiphyte Hosts												Total
		1-28b	13P - 4(2) II	13P - 5(1) II	17P -3-2 II	21-1+2	21+5-1	2+5	39C II	39L II 2	39U II	39V II	39WII	
NA	ΦEa 9-3													10
	ΦEa 10-1													10
	ΦEa 10-5													12
	ΦEa 10-14													2
	ΦEa 10-17													8
	ΦEa 45-1A													2
	ΦEa 45-1B													9
	ΦEa 45-3													3
	ΦEa 46-1A1													4
	ΦEa 46-1A2													9
	ΦEa 46-1A3													8
	ΦEa 46-1C													5
	ΦEa 46-1E													2
	ΦEa 50-3								NT	NT	NT	NT		≥ 3
	ΦEa 51-8A													6
ΦEa 51-8B													9	
ΦEa 51-8C													12	
1	ΦEa 10-2													11
	ΦEa 10-3													8
	ΦEa 10-4													7
	ΦEa 21-1								NT	NT	NT	NT	NT	≥ 5
	ΦEa 21-2													8
	ΦEa 21-3													10
ΦEa 21-4													11	
2	ΦEa 10-6													9
	ΦEa 31-2													5
	ΦEa 31-4													4
	ΦEa 35-2													10
	ΦEa 35-4													11
	ΦEa 35-6													10
ΦEa 35-7													7	
3a	ΦEa 10-7													0
	ΦEa 10-8													1
	ΦEa 10-9													2
	ΦEa 10-10													4
	ΦEa 10-11													5
	ΦEa 10-13													5
	ΦEa 10-15													1
ΦEa 31-3													10	
3b	ΦEa 46-2													3
3c	ΦEa 1													12
4	ΦEa 10-12													8
	ΦEa 10-16								NT	NT	NT	NT	NT	≥ 4
	ΦEa 35-3													10
	ΦEa 35-5													7
5	ΦEa 9-2													1
	ΦEa 9-4													10
	ΦEa 9-5													10
6	ΦEa 51-1						NT							6
	ΦEa 51-2													7
	ΦEa 51-3													4
	ΦEa 51-4													5
	ΦEa 51-6		NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NA
ΦEa 51-7													1	
Total		24	25	27	27	27	28	32	26	34	29	32	35	

but less than the more concentrated treatments, all of which caused at least 90% disease ( $P < 0.05$ ). Results similar to this second experiment were also obtained when the *E. amylovora* suspensions were applied using a custom-made air pressure-driven atomizer at a spray rate of 15 to 70  $\mu\text{L}/\text{cm}^2$ .

The carrier candidates that were screened for susceptibility to phage infection in the Phase 2 screening were also tested in blossom assays. Very few of them had any effect on the development of fire blight symptoms. Only blossoms treated with 39CII or 39LII2 showed a significant reduction in disease severity ( $P < 0.05$ ) 4 d after inoculation with *E. amylovora*, ranging from 34 to 45% less diseased surface area than the untreated controls. Blossoms treated with Eh21-5 and 39UII showed 21 and 13% reductions in symptom severity, respectively, though these were not statistically significant.

Very few phages caused a noticeable reduction in symptom severity, and statistically significant reductions in disease severity were only observed for  $\phi\text{Ea}10\text{-}1$ ,  $\phi\text{Ea}10\text{-}6$ ,  $\phi\text{Ea}10\text{-}7$ ,  $\phi\text{Ea}10\text{-}8$  ( $P < 0.05$ ). Other phage treatments resulted in a mean symptom severity less than that of buffer treated blossoms, even though the difference was not statistically significant: from group 1,  $\phi\text{Ea}21\text{-}3$ ,  $\phi\text{Ea}21\text{-}4$ ; from group 2,  $\phi\text{Ea}10\text{-}6$  and  $\phi\text{Ea}35\text{-}4$ ; of the group 3 phages,  $\phi\text{Ea}10\text{-}15$  and  $\phi\text{Ea}31\text{-}3$ ; from group 4,  $\phi\text{Ea}35\text{-}5$ ; from group 5,  $\phi\text{Ea}9\text{-}5$ ; from group 6,  $\phi\text{Ea}51\text{-}1$ , and  $\phi\text{Ea}51\text{-}2$ ; of the ungrouped phages,  $\phi\text{Ea}10\text{-}1$ ,  $\phi\text{Ea}45\text{-}1\text{B}$ , and  $\phi\text{Ea}46\text{-}1\text{A}2$ . There was a general trend towards less severe symptom development in blossoms treated with an ungrouped phage.

## Discussion

A selection of molecular and morphological, and infection characteristics of the Vineland collection are presented here.

Molecular analyses indicate that the revived phage collection is not identical to the original collection (Gill, 2000; Gill et al., 2003). The RFLP patterns of some isolates indicate that the stocks became mixed, and the original phage was not re-isolated during revival. Some of the stocks exhibit plaque morphologies, and even RFLP patterns, that are consistent with the isolate description, even though an PCR amplicon was produced based on the  $\phi$ Ea1 primers designed by Schnabel and Jones (2001). This latter case suggests that the stocks may be mixed, with the group 3 phage present in sufficient concentration to be detected by PCR. Comparisons between the current collection and previous descriptions (Gill, 2000; Gill et al., 2003) should be drawn with care.

There were some differences among the results of the three PCR assays (Table 2-3), but most strong real-time PCR signals were obtained from phage stocks that also gave a positive result with the  $\phi$ Ea1 primers. The differential success of the two real-time PCR assays indicates variation in the depolymerase gene sequence between phages that apparently possess the gene. The fact that some phages could not be amplified by any of the three PCR assays (Table 2-3) is consistent with other indicators of the diversity of the Vineland collection. All are based on sequence data from  $\phi$ Ea1, and while many *E. amylovora* phages are *Podoviridae* similar to  $\phi$ Ea1, there is no reason to expect that all of them share substantially similar genomes. The EPS-degrading enzyme on which the real-time PCR detection was based is not even common to all *Podoviridae* of Enterobacteriaceae (Geller et al., 1998). Real-time PCR will eventually be used

to quantify the populations of phage, carrier, and pathogen during field trials, and to track the environmental fate of the phages after their application. Not all of the phages selected for use in field trials need to be detectable by these real-time PCR primers and probes, but some of them do so that the population dynamics of the phages can be related to those of the carrier and pathogen, as well as to the overall efficacy of a given treatment.

The host range of the Vineland collection on *E. amylovora* strains was slightly different from that reported by Gill (2000), which is consistent with the results of the collection revival and the molecular characterization data. However, the characteristic host range patterns of group 3 and group 6 phages were similar to those described previously. Group 3 phages belong to *Podoviridae*, and produce depolymerase, as evidenced by the expanding halo around each plaque. Most of these phages only infected the *E. amylovora* strains that produce noticeable amounts of EPS even on media that did not contain any added sucrose. The group 4, group 6, and some of the ungrouped phages are also members of the *Podoviridae* (Gill et al., 2003). Of these, only the group 6 phages showed this same strong bias towards infecting strains with abundant EPS. However, none of the group 4 or ungrouped phages failed to infect those same strains, whereas some phages in the other groups did.

With the exception of phage S1 (Erskine, 1973), none of the previously described *E. amylovora* phages were reported to infect other bacterial species, even the closely related *P. agglomerans* (Ritchie & Klos, 1979; Schnabel & Jones, 2001). Very different results were obtained in the Phase 2 carrier screens conducted here, where many *P. agglomerans* isolates were infected by multiple phages (Table 2-7). Previous studies have used on a single bacterial host strain in their phage isolation protocols and have mostly recovered  $\phi$ Ea1-like phages, which did



exhibit narrower host ranges in this study as well. Previous studies also tested only 1 to 3 strains of the species in question. Even within *E. amylovora* there are strains which are resistant to infection by many or most of the phages that will infect other strains.

Most phages are highly specific to one or a few bacterial species, with a few notable exceptions. ø-S1 infects multiple strains of at least 17 different *Pseudomonas* species and biotypes (Kelln & Warren, 1971). P1 infects multiple enteric species (Yarmolinsky & Steinberg, 1988). Phage mu also has a broad host range, in this case as the result of a genetic switch that introduces variation to the structure of its tail fibres (van de Putte, Cramer, & Giphart-Gassler, 1980; Grundy & Howe, 1984; Plasterk, Kanaar, & van de Putte, 1984). The host range data collected in this study show the Vineland phages to be more promiscuous than most phages are thought to be, but still generally limited to closely related species and genera.

The phages used in this work were isolated from orchards and gardens and so would not be expected to greatly disrupt the microbial ecology of an orchard to which they were exogenously applied. Nevertheless, it is important to know what other orchard bacteria might be affected by their presence. The specificity of phages is an advantage in this respect, though the Vineland collection exhibits a broader host range than many phages, infecting both *E. amylovora* and *P. agglomerans*. The orchard bacteria tested here are by no means an exhaustive sample of orchard microflora, but the resistance of the tested isolates to phage infection indicates that the effect of these *E. amylovora* phages on other orchard bacteria would be minimal to non-existent. The susceptibility of *P. syringae* pv. *papulans* psp mutsu to *E. amylovora* phages is suspicious, given the phylogenetic distance between the Enterobacteriaceae and the Pseudomonadaceae. However, there was a striking similarity in the identity of phages that infected this isolate and *P.*

*agglomerans* E325, which suggests that the original identification of the *P. syringae* pv. *papulans* psp mutsu isolate as *Pseudomonas* may be inaccurate.

Antibiotic production was observed in all of the phase 2 carrier candidates. Several of the carrier candidates produced an antibiotic that may be similar to pantocin B, based on the large, diffuse zone of inhibition produced by the metabolites of all of these strains. Unfortunately the biosynthetic pathway responsible for the production of pantocin B has not been characterized, and so no PCR screen is available to confirm the identity of this antibiotic. Inactivation tests could be used to support or refute this identification, since pantocin B is inactivated by histidine and by an extracellular protease made by *P. fluorescens* A506. Mass spectrometry could also be used to compare these antibiotics to pantocin B, following purification of the active soluble species by liquid chromatography.

Part of the pantocin A biosynthetic gene was present in Eh252, C9-1, 39CII, and 1-28b. The first three of these strains produced zones of inhibition characteristic of pantocin A (Ishimaru et al., 1988). Strain 1-28b did not produce this zone of inhibition, even though the *paaB* gene fragment was amplified. Since pantocin A is produced by a complex biosynthetic process (Jin, Wright, Beer, & Clardy, 2003), it is possible that the *paaB* gene is present in the 1-28b genome, but that the biosynthetic pathway is not complete.

The antibiotic produced by 13P-4-5(2)II strongly inhibits *E. amylovora* strains, and is not similar to a previously described antibiotic. If this antibiotic is not inactivated by any of the amino acids that are usually found in pear or apple nectar (Lewis, Tolbert, & Kenworthy, 1964) then it would likely be active on the blossom surface, and may allow strain 13P-4-5(2)II to inhibit *E. amylovora* very effectively.

Because of the non-quantitative nature of the filter disc assay, conclusions can not be drawn regarding the relative susceptibilities of different *E. amylovora* strains to these antibiotics. Comparative susceptibility tests could be done using volumes of growth medium that are standardized based on the final cell density in the culture. Rather than reducing the volumes of culture filtrate from lower density cultures, the total amount of the antibiotic present in the plugs can also be increased by allowing the disc to dry slightly before applying an additional volume of the culture filtrate. This is more likely to allow detection of the weaker antibiotics such as pantocin B.

The *in planta* assay used to screen the phages and carrier candidates for their ability to inhibit *E. amylovora* was a slightly modified version of the blossom assay described by Gill (2000). Assays based on apple and pear blossoms have been used previously to assess *E. amylovora* pathogenicity, test the efficacy of biological control agents, and study the effects of blossom nutrition on blossom chemistry and microbial growth (Pusey, 1997; Gill, 2000; Pusey & Curry, 2004; Johnson, Stockwell, & Sawyer, 2004). Blossom assays are based on the fact that most fire blight outbreaks begin with blossom infection, and thus biopesticide efficacy depends on the microbial ecology of the blossom. Other commonly used bioassays for the pathogenicity and biological control of *E. amylovora* are the pear plug bioassay and infection of seedling shoot tips with scissors dipped in bacterial suspensions. The pear plug assay is based on the growth of *E. amylovora* and the development of a characteristic ooze on the surface of immature pear fruit tissue. The seedling assay is meant to mimic the infection of a succulent shoot during insect feeding. While these are both convenient screening methods, and *E. amylovora* does infect both fruit tissue and shoots, neither of these assays reflect the primary mode of host tissue infection,

and certainly does not reflect the characteristics under which the phage-carrier biopesticide is expected to function.

The selection of phages and the carrier was not entirely based on their efficacy in blossom assays. Since the carrier must be infected by the chosen phages, the selection of each is dependent on the selection of the other. Very few phages consistently caused a noticeable reduction in symptom severity. In retrospect, this may be largely attributable to an unnecessarily high pathogen pressure. Initial tests had indicated that a  $1 \times 10^8$  CFU/mL suspension of *E. amylovora* was needed to cause full disease in at least 80% of blossoms, but all inoculum tests conducted later have shown that the same level of disease is possible with a  $1 \times 10^6$  CFU/mL suspension. The latter result is considered to be more accurate given the larger sample size used, the finer gradations of pathogen inocula tested, and the greater repeatability of the results. Therefore the phages and carrier were selected from among those candidates that gave notable protection from fire blight symptoms in the blossom assays, even if that difference was not statistically significant.

From the phages within each RFLP group, one or two were selected that appeared to have been the most effective in the blossom assays, and that infected at least six of the carrier candidates during the Phase 2 screening. Very few of the effective group 3 phages had a broad enough host range in *P. agglomerans* isolates to be useful. The Phase 2 carrier candidates were then considered in terms of how many of these phages infected it, and its performance in the blossom bioassay. This should have balanced phage selection so that a genetically diverse group of effective phages is tested, which can later be formulated in to diverse cocktails to reduce the risk of selecting for phage-resistant *E. amylovora*.

*P. agglomerans* isolate Eh21-5 was selected as the bacterial carrier for those field trials based on its biocontrol ability in blossom assays, and its susceptibility to a wide range of bacteriophages. This strain also produces a weak antibiotic, but it is not known whether this antibiotic is active on the blossom surface. *P. agglomerans* 13P-4-5(2)II is a promising backup candidate, since it is infected by many of the same phages as Eh21-5 and produces a strong, novel antibiotic. Ten phages,  $\phi$ Ea9-5,  $\phi$ Ea10-1,  $\phi$ Ea10-6,  $\phi$ Ea 21-4,  $\phi$ Ea31-3,  $\phi$ Ea35-4,  $\phi$ Ea35-5,  $\phi$ Ea45-1B,  $\phi$ Ea46-1A2, and  $\phi$ Ea51-1, were chosen for further development and for use in field trials based on their performance in blossom assays, genetic diversity as indicated by RFLP groupings and host range in potential *P. agglomerans* carriers, and detectability by the real-time PCR primers designed for determining phage numbers in the orchard. Three of these,  $\phi$ Ea9-5,  $\phi$ Ea35-5 and  $\phi$ Ea51-1 were later dropped from the development program.

Seven phages and *P. agglomerans*Eh21-5 were selected for further study toward the development of the phage-carrier biopesticide for fire blight.

### Chapter 3: The Complete Genome Sequence of *Erwinia* phage $\phi$ Ea21-4

#### Abstract

Very little genome sequence information is available from which to develop PCR-based detection tools for *Erwinia* phages. In order to address this shortage of information, the complete genome of  $\phi$ Ea21-4 was sequenced to an average PHRAP 40 quality, from a shotgun genomic library, followed by primer walking. The single-copy genome is 84.7 kb in length, with a GC content of 43.8%. The packaged genome is terminally redundant. Eighty-two ORFs were predicted using GeneMark.hmm, a trained Markov-chain model. Since all phage ORFs are expected to be translated, preliminary annotation was conducted by comparing predicted protein sequences to all possible translations of the global nucleotide database. The  $\Phi$ Ea21-4 genome appears to be substantially different, both globally and locally, from previously reported sequences. About 31% of predicted ORFs were assigned a putative function based on amino acid sequence comparisons. Four more ORFs were identified as structural proteins based on SDS-PAGE of denatured phage particles and N-terminal sequencing of the isolated proteins. In each case, the complete protein sequence was highly similar to a previously unidentified protein from *Salmonella* phage Felix 01. No notable similarity exists between the  $\Phi$ Ea21-4 genome and previous sequences from the  $\Phi$ Ea1(h) and Era103 genomes. Only the Felix genome shows any broader similarity to  $\Phi$ Ea21-4, and even this consists of only 40% to 50% sequence identity across less than 20% of the two genomes. No significant similarity to available sequences was found for 23% of the predicted ORFs.

## Introduction

About 5300 different phages have been described (Ackermann, 2006). They are classified based on the type of nucleic acid genome (single- or double-stranded RNA or DNA), the number of nucleic acid segments per genome, morphology (binary, cubic, or helical symmetry; pleiomorphy), the presence and nature of a lipid membrane, whether they are capable of lysogeny, and the mechanism by which mature progeny are released from the host cell. Approximately 96% of known phages belong to the order Caudovirales, the tailed phages (Ackermann, 2001). This does not necessarily reflect the relative frequency of Caudovirales species in nature. It is estimated that less than 0.0002% of Earth's phage metagenome has been described (Rohwer, 2003; Guttman, Raya, & Kutter, 2005), and most detailed work has been conducted on phages of Gram-negative bacteria, particularly phages of the Enterobacteriaceae since these bacteria frequently cause disease in humans and other animals. As a result, the list of described phages is likely biased in favour of those types that infect the most commonly studied and easily cultured host organisms.

The Caudovirales order is divided into three families based on shared morphological features: *Siphoviridae*, *Myoviridae*, and *Podoviridae*. The *Siphoviridae* have long, flexible, non-contractile tails, and comprise about 61% of observed tailed phages. The *Myoviridae* have contractile tails made up of a sheath and central tube, and account for about 25% of observed tailed phages. The remaining 14% of Caudovirales phages are identifiable by their extremely short tails, and belong to the *Podoviridae* family. All of these phages have double-stranded DNA genomes, and are composed of about 50% protein and 50% nucleic acid (Ackermann, 2001).

Phages that infect *E. amylovora* have been isolated from soil since the 1950s (Okabe &

Goto, 1963), but detailed characterization only began in the 1970s. Selected characteristics of these described phages are compiled in Table 3-1. Phages S1 (Erskine, 1973) and Era103 (Vandenbergh, Wright, & Vidaver, 1985) appear to be unique, but were the sole phages discussed in their respective publications. More useful information as to the diversity of *E. amylovora* phages can be culled from larger studies in which many phage isolates were collected. Schnabel and Jones (2001) collected 50 phage isolates from apple and pear orchards in Michigan and California, and a raspberry farm in Michigan. Forty-two of the isolates from Michigan and California tree fruit orchards could not be distinguished from  $\phi$ Ea1. Four isolates from raspberry and Michigan apple orchards were indistinguishable from  $\phi$ Ea7. Only four isolates appeared to be novel phages, and two of these,  $\phi$ Ea100 and  $\phi$ Ea104 were quite similar. Gill et al (2003) reported a more diverse collection of isolates. These were classified by RFLP patterns and  $\phi$ Ea1-based PCR. Ten phages, groups 3a and 3b, were related, but not identical, to  $\phi$ Ea1. Ten isolates were not placed into the reported groups, either because they produced a completely unique RFLP pattern or could not be digested by any of the endonucleases used. The remaining 30-plus isolates were distributed much more evenly among the different RFLP groups than the isolates collected by Schnabel and Jones (2001), and it would be premature to state that each RFLP group constitutes a single phage strain, since electron microscopy and further molecular analysis have revealed some differences between isolates within a group (see Tables 2-3 and 2-4). The greater diversity of the Vineland collection may be due to use of six *E. amylovora* strains during the enrichment and isolation process. Other studies have only used a single host strain, which would bias the enrichment process in favour of those phages that infect that strain most efficiently, or that produce the largest burst size from each infected cell (Jensen et al., 1998).



Table 3-1. Characteristics of previously described *E. amylovora* phages. Except for Era103, genome size estimates are minimums based on PFGE and RFLP data.

Phage	Isolated from	Host Range (other than <i>E. amylovora</i> )	Family	Approximate Genome Size (kb)	Reference <sup>a</sup>
S1	soil beneath infected pear	<i>P. agglomerans</i> <sup>b</sup>	Caudovirales, icosahedral head	- <sup>c</sup>	(Erskine, 1973)
Era103	infected apple and pear tissue	not <i>P. agglomerans</i>	<i>Podoviridae</i>	45	(Vandenbergh et al., 1985; Summer et al., 2007) GenBank: EF160123
φEa1 <sup>d</sup>	infected apple tissue	not <i>Pseudomonas</i> or <i>P. agglomerans</i>	Caudovirales, polyhedral head	46	(Ritchie & Klos, 1979; Schnabel & Jones, 2001)
φEa7	infected apple tissue	not <i>Pseudomonas</i> or <i>P. agglomerans</i>	Caudovirales, octahedral head	35	(Ritchie & Klos, 1979; Schnabel & Jones, 2001)
φEa100	soil beneath infected apple	NT <sup>c</sup>	-	35	(Schnabel & Jones, 2001)
φEa104	soil beneath infected apple	NT	-	35	(Schnabel & Jones, 2001)
φEa125	soil beneath infected apple	NT	-	35	(Schnabel & Jones, 2001)
φEa116C	infected apple tissue	not <i>Pseudomonas</i> or <i>P. agglomerans</i>	-	75	(Schnabel & Jones, 2001)
Group 1 (9 isolates)	infected apple tissue and soil beneath infected pear	<i>P. agglomerans</i> ; not <i>Pseudomonas</i> or <i>E. coli</i>	<i>Myoviridae</i>	75	(Gill, 2000; Gill et al., 2003)
Group 2 (4 isolates)	various	NT	<i>Myoviridae</i>	75	(Gill, 2000; Gill et al., 2003)
Group 3a (9 isolates)	soil beneath infected apple	not <i>Pseudomonas</i> , <i>E. coli</i> , or <i>P. agglomerans</i>	<i>Podoviridae</i>	36	(Gill, 2000; Gill et al., 2003)
Group 3b (1 isolate)	infected crabapple tissue	<i>P. agglomerans</i> ; not <i>Pseudomonas</i> or <i>E. coli</i>	<i>Podoviridae</i>	45	(Gill, 2000; Gill et al., 2003)

Phage	Isolated from	Host Range (other than <i>E. amylovora</i> )	Family	Approximate Genome Size (kb)	Reference
Group 4 (2 isolates)	soil beneath infected apple	<i>P. agglomerans</i> ; not <i>Pseudomonas</i> or <i>E. coli</i>	<i>Podoviridae</i>	67	(Gill, 2000; Gill et al., 2003)
Group 5 (3 isolates)	soil beneath infected pear	NT	<i>Podoviridae</i>	45	(Gill, 2000; Gill et al., 2003)
Group 6 (6 isolates)	soil beneath infected Rosaceae	<i>P. agglomerans</i> ; not <i>Pseudomonas</i> or <i>E. coli</i>	<i>Podoviridae</i>	62	(Gill, 2000; Gill et al., 2003)
Various (10 isolates)	mostly soil beneath infected apple or pear	NT	various	-	(Gill, 2000)

<sup>a</sup> References are to the publication(s) from which the phage's description is taken, which is not necessarily the original report of its isolation.

<sup>b</sup> In several of these studies, *P. agglomerans* is referred to by its former name, *Erwinia herbicola*.

<sup>c</sup> “-” indicates that no information about the trait was reported.

<sup>d</sup> Also referred to as  $\phi$ Ea1(h) in Ritchie and Klos (1979). The phage designated  $\phi$ Ea1(nh) was a derivative of  $\phi$ Ea1(h) that was never isolated directly from plant material.

<sup>e</sup> “NT” indicates that the host range was not tested with any species other than *E. amylovora*.

PCR primers designed based on the sequenced region of  $\phi$ Ea1 failed to amplify about 50% of the phages in Vineland collection (Chapter 2). This, combined with the data from RFLP analysis and transmission electron microscopy (Chapter 2; Gill, 2000), indicate that there is a substantial amount of genetic diversity among *E. amylovora* phages that is not reflected by the currently available sequence data. In order to take full advantage of this diversity during biopesticide development, more genomic information is needed in order to understand it. To that end, the complete genome of  $\phi$ Ea21-4 was sequenced and annotated.  $\phi$ Ea21-4 is a tailed, contractile *E. amylovora* phage that was isolated in 1998 from a pear orchard in southern Ontario, Canada. It performed well in preliminary biological control assays, but was not detected by primer and probe sets based on the  $\phi$ Ea1 depolymerase gene (see Table 2-3).

The National Center for Biotechnology Information (NCBI) genome database currently contains complete genome sequences for almost 400 bacteriophages, and yet, at the time this work was undertaken, the only sequence data available was a 3.3 kb region of  $\phi$ Ea1 (Kim, Salm, & Geider, 2004). Early in 2007, the complete genome sequence of *E. amylovora* phage Era103 was deposited (Summer et al., 2007).

## Methods

### *Strains and Growth Conditions*

*E. amylovora* phage  $\phi$ Ea21-4 was grown on *E. amylovora* Ea6-4 in liquid culture, as described in Chapter 2.

### *Transmission Electron Microscopy*

A concentrated suspension of *E. amylovora* phage  $\phi$ Ea21-4 was prepared by centrifuging 10 mL of filtered lysate at 16 000  $\times g$  for 75 min, decanting the supernatant and resuspending the phage pellet in 5 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA. TEM work was conducted as described in Chapter 2.

### *DNA isolation and RFLP*

Three methods of DNA isolation were compared: the CTAB and organic extraction methods previously described (see Chapter 2), and the Qiagen lambda mini kit (Qiagen, Mississauga, ON). DNA extraction using the Qiagen kit was conducted according to the manufacturer's protocol, except that the phage precipitation step was doubled to 2 h. An RFLP was conducted using each of *MvnI*, *BglII*, *BamHI*, and *EcoRI*, as described in Chapter 2.

### *Genome Sequencing*

The DNA from multiple organic extractions was combined and the concentration was determined based on the absorbance of ultraviolet light ( $\lambda = 260$  nm, 50  $\mu\text{g/mL}$  = OD of 1). A composite sample was prepared by combining extraction products with an absorbance ratio (260

nm vs. 280 nm) between 1.7 and 1.9 and adjusting the concentration to approximately 100  $\mu\text{g/mL}$ . Approximately 100  $\mu\text{g}$  of DNA was sent to Agencourt Bioscience (Beverly, MA) for shotgun cloning and sequencing. The shotgun genome library was constructed by mechanically shearing the  $\phi\text{Ea21-4}$  DNA. The resulting 3 to 4 kb fragments were cloned into the proprietary pAGEN vector using the BstXI adaptor. *Escherichia coli* colonies carrying the cloned vectors were selected and inserts sequenced for 10X coverage. Sequencing reactions were conducted using an ABI PRISM 3730xl DNA Analyzer and BigDye Terminator v3.1 reagents.

### *Sequence Analysis*

Putative open reading frames (ORFs) were identified using GeneMark.hmm, version 2, and Fickett's TESTCODE, and the ORFs predicted by both models were accepted. GeneMark is a Markov chain model trained on completed microbial genomes (Besemer, Lomsadze, & Borodovsky, 2001). Fickett's TESTCODE method is based on asymmetrical base usage in coding regions and is built into the Clone Manager Professional Suite program (v. 7.11, SciEd Central). Similarity to described genes in the global database (available through NCBI at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) was tested using the tblastn algorithm, which compares protein translations of the predicted ORFs to all possible translations of the nucleotide database.

Two programs were used to search for phage-encoded tRNAs: tRNAscanner (Lowe & Eddy, 1997), and FAStrRNA (El-Mabrouk, & Lisacek, 1996). Loci predicted by both programs were accepted.

Genome-wide comparisons were made using the Genome Shovel dot-plot program, available from the Japan Science and Technology Agency ([www-btls.jst.go.jp](http://www-btls.jst.go.jp)). The genomes

were aligned using the tblastx algorithm, and are displayed as DNA.

Two-way protein alignments were constructed using ALIGN Query, available from the Genestream Search network server IGH in Montpellier, France ([xylan.igh.cnrs.fr/bin/align-guess.cgi](http://xylan.igh.cnrs.fr/bin/align-guess.cgi)). Protein domain searches were conducted using the Pfam 21.0, available from the Sanger-Wellcome Trust (Finn et al., 2006)

The 3.3 kb fragment of  $\phi$ Ea1 and the complete genomes of  $\phi$ Era103 and Felix were downloaded from the NCBI GenBank database (AJ278614, NC\_009014, and NC\_005282, respectively).

### *Identification of Major Structural Proteins*

Intact phage particles were purified using cesium-chloride gradient centrifugation, as previously described (Sambrook & Fritsch, 1989). Syringe-filtered  $\phi$ Ea21-4 lysate was concentrated by centrifugation at 16 000 xg for 1 h and resuspended in 1/10th volume of SM buffer. Bacterial nucleic acids were digested by adding 1  $\mu$ L each of 10 mg/mL DNase I and RNase A, and incubating the reaction at 37°C for 30 min. Approximately 4 mL of this phage suspension ( $2.1 \times 10^{10}$  PFU/mL) was dissolved in sterile distilled water with CsCl to a final density of 1.48 g/mL in a final volume of approximately 23 mL. The suspension was divided evenly between two Beckman OptiSeal 11.2 mL polyallomer ultracentrifuge tubes (Beckman Coulter Canada, Mississauga, ON). The suspension was centrifuged at 30,000 rpm for 24 h using the NVT 65 rotor on a Beckman Optima CL-100K ultracentrifuge. Following centrifugation, the phage particles were visible as an opaque band. The centrifuge tube was pierced just below the band and the phages were drawn off using a 26 ½-gauge hypodermic needle with a 1 mL syringe.

Cesium chloride was removed by dialysis using four changes of buffer (10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, pH 8.0), lasting 15 min each. A second batch of purified phage was prepared in the same way, with the following changes: the syringe-filtered phage lysate contained  $3 \times 10^{11}$  PFU/mL, and the gradient-purified phage were drawn from the CsCl using an 18-gauge needle.

The phage suspension was concentrated by freeze-drying. A 2 mL microcentrifuge tube containing 0.5 mL phage suspension was capped with a 1" square of kimwipe and immersed in liquid nitrogen for approximately 30 s, until frozen. Samples were vacuum-dried overnight using a ThermoSavant MicroModulyo (E-C Apparatus, Holbrook, NY).

The sample was resuspended in 100  $\mu$ L of 2X gel loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.1 mg/L bromphenol blue). Proteins were denatured by immersing the sample in a boiling water bath for 5 min. Proteins were separated by SDS-PAGE. Twenty-five microliters of the concentrated sample, and 10  $\mu$ L of BenchMark pre-stained protein ladder (Invitrogen) was loaded on each of two 12% SDS-PAGE gels, prepared according to published protocols (Sambrook & Fritsch, 1989) and run at 150V for 70 min. One gel was stained overnight in Coomassie blue (0.1% (w/v) Coomassie blue R-250, 40% (v/v) methanol, 10% (v/v) glacial acetic acid), and destained in 40% (v/v) methanol with 10% (v/v) glacial acetic acid, and observed at various times during destaining. The gel was photographed with a Kodak Easy-Share CX7430 digital camera, through a SYBR Safe photographic filter (Wratten filter No. 9, Molecular Probes, Eugene, OR) filter.

The proteins were transferred from the second gel to a polyvinyl difluoride (PVDF) membrane using the mini-Protean II transfer cell (BioRad Laboratories, Hercules, CA). The

PVDF membrane was prepared by brief immersion in methanol, and the gel, membrane, and filter paper were equilibrated in Towbin transfer buffer (3.03 g/L Tris base, 14.41 g/L glycine, 20% (v/v) methanol) for 15 min. The electroblot sandwich was assembled as described by Sambrook and Fritsch (1989) and protein transfer was conducted overnight at 30 V. The separated proteins were visualized by immersing the membrane in Coomassie blue for 10 min, and destaining in several rinses of 50% (v/v) methanol for a total of approximately 15 min. Stained bands were excised from the membrane using a razor blade, placed in microcentrifuge tubes, and stored at 4°C. N-terminal Edman microsequencing was conducted by the Advanced Protein Technology Centre at the Hospital for Sick Children. N-terminal amino acid sequences were compared to the hypothetical translated sequences of all predicted ORFs in the  $\phi$ Ea21-4 genome.

In order to separate the two proteins with an apparent molecular size of 42 kDa, a 1 mL volume of the CsCl-purified phage suspension was separated by isoelectric focusing. The Rotofor isoelectric focusing system (BioRad) was assembled, loaded, and run according to the manufacturer's instructions, using the standard focusing chamber. The sample was prepared by combining 58 mL distilled water, 1 mL of purified phage suspension that had been denatured at 100°C for 5 min, and 3 mL of Bio-Lyte ampholytes (pH range 3/10). The fractionation run was conducted at 15 W for approximately 2 h, and the resulting fractions were collected in 20 12 x 75 mm culture tubes. The fractions were transferred into 2 mL microcentrifuge tubes and freeze-dried as previously described. The lyophilized fractions were resuspended in 1X gel-loading buffer and run on a 12% SDS-PAGE gel as previously described.



## Results

### *Morphological Features of $\phi$ Ea21-4*

$\phi$ Ea21-4 is a tailed, contractile phage belonging to the *Myoviridae* family, and as such has a double-stranded DNA genome. Figure 3-1 shows the contracted and uncontracted states of the  $\phi$ Ea21-4 virion. The head is icosahedral, and approximately 60 nm across, with a 90 nm tail. In the uncontracted state, a slight narrowing of the proximal end of the tail, called the neck, is visible above the tail sheath where the central tube connects to the head. In the contracted state, the central tube can be seen extending past the base of the contracted sheath, and the neck and base plates are visible above and below the sheath, respectively.

### *DNA Extraction*

The organic solvent extraction method was the best of the three DNA purification methods tested. Table 3-2 shows the relative purity of DNA obtained from each method. DNA extracted by the CTAB method had the lowest and most variable purity. The organic and kit extraction methods yielded DNA of similar purity, but results were more consistent with the organic extraction method. In addition, the kit method rarely yielded detectable amounts of DNA, whereas DNA was consistently recovered using the organic extraction method.

During later organic extractions it was noted that the addition of the usual 2.0 to 2.5 volumes of 100% ethanol during DNA precipitation resulted in the precipitation of a dense, viscous material, in which flecks of precipitated DNA were embedded. This substance was not precipitated from the aqueous phase of back-extracted DNA, in which fresh Tris-HCl was added to the used phenol phase, extracted as usual, added to the used phenol-chloroform phase,

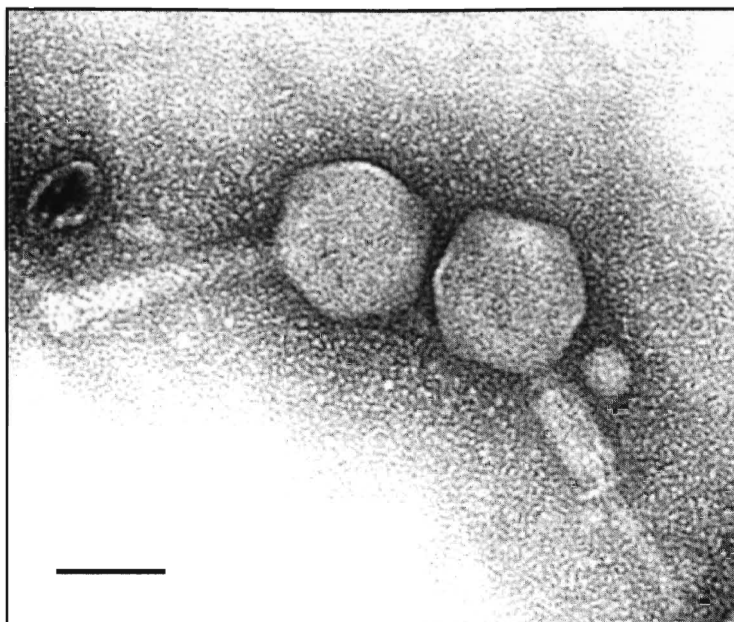


Figure 3-1. TEM of  $\phi$ Ea21-4. The phage is shown here in the uncontracted (left) and contracted states (right). [micron marker = 50 nm]

Table 3-2. Effect of extraction method on the A260/A280 ratio of genomic  $\phi$ Ea21-4 DNA.

Method	Absorbance(260nm)/Absorbance(280nm)		
	mean <sup>a</sup>	SD	range
Organic Extraction (n = 3)	1.94 <sup>A</sup>	0.02	1.92 - 1.95
Qiagen kit (n = 3)	1.91 <sup>A</sup>	0.17	1.66 - 2.13
CTAB (n = 6)	1.52 <sup>B</sup>	0.32	1.00 - 1.82

<sup>a</sup> Means with the same letter are not significantly different (Tukey test for mean separation,  $\alpha = 0.05$ )

extracted, etc. to recover DNA lost to the organic phases in the original extraction. The use of fresh extraction reagents did not eliminate the problem. The ultraviolet absorbance spectrum (200 to 320 nm) of this viscous substance was characteristic of carbohydrate, with a peak at 225 nm, and very little absorbance above 240 nm. In an attempt to precipitate the carbohydrate fraction separately from the dissolved DNA, ethanol was added to the final aqueous phase 100  $\mu$ L at a time, mixing by inversion after each addition. After an equal volume of ethanol had been added to the final aqueous phase, the DNA precipitated, but the carbohydrate did not. The precipitated DNA could be sedimented, dried, and resuspended as usual from this point, and the purity of the resulting sample was comparable to that indicated in Table 3-2.

### *Genome Sequencing*

Shotgun sequencing yielded a 30 kb contig and nine non-overlapping inserts, for a total of approximately 39 kb of sequence data. Since the total length of these sequences was only half the 75 kb genome size estimated from RFLP analysis (Gill, 2000), the sequencing company was requested to begin primer walking from the ends of each contig. After nine rounds of primer walking, a single contig of 80 kb was obtained. After two more rounds of terminal primer walks, the same 182 bp sequence was obtained from both reactions and primer walking was halted. The final sequence was 84.7 kb for the single-copy genome, which means that slightly more than 50% of the genome was not represented in the random clone library.

### *General Features of the $\phi$ Ea21-4 Genome*

The genome sequence obtained from Agencourt matches the known sequence

characteristics of the DNA isolated from  $\phi$ Ea21-4 lysate. Figure 3-2 shows the RFLP patterns that were obtained by digesting genomic  $\phi$ Ea21-4 DNA with each of *SalI*, *BamHI*, *BglII*, and *EcoRI*. The fragment sizes predicted by virtual restriction digests of the sequence were consistent with these RFLP patterns, confirming that the sequenced genome matched the known molecular characteristics of  $\phi$ Ea21-4. Also, the sequences of the  $\phi$ -dpo1 primers and probe (see Chapter 6), which did not amplify DNA from the  $\phi$ Ea21-4 lysate, were not found in the genome sequence.

The  $\phi$ Ea21-4 genome is quite different from  $\phi$ Ea1 and  $\phi$ Era103. The region surrounding the  $\phi$ Ea21-4 endolysin gene was compared to the 3.3 kb sequence from  $\phi$ Ea1. The only region that could be meaningfully aligned was that of the endolysin gene itself, and then with only 32% sequence identity at the amino acid level (Figure 3-3). In addition, none of the primers or probes designed from the *dpo* gene of  $\phi$ Ea1 by Dr. Kim (see Chapter 2) match regions of the  $\phi$ Ea21-4 genome with more than 50% identity, and none of the partial matches could reasonably permit PCR amplification. The only other complete genome sequence available for a *E. amylovora* phage is  $\phi$ Era103. A dot-plot comparing the  $\phi$ Ea21-4 and  $\phi$ Era103 genomes was constructed (Figure 3-4). Even using low identity thresholds, there was very little similarity between the two genomes.

The only phage genome with notable similarity to  $\phi$ Ea21-4 genome is that of *Salmonella* phage Felix 01. Figure 3-5 is a dot-plot comparing the genomes of  $\phi$ Ea21-4 and Felix. Using a 100 bp sliding window, no regions of 65% or greater identity were observed. Regions totaling perhaps 15-20% of the genomes were 40-65 % identical. These regions fell mostly between bases 12,000 and 40, 000, or between 63,000 and 70,000.

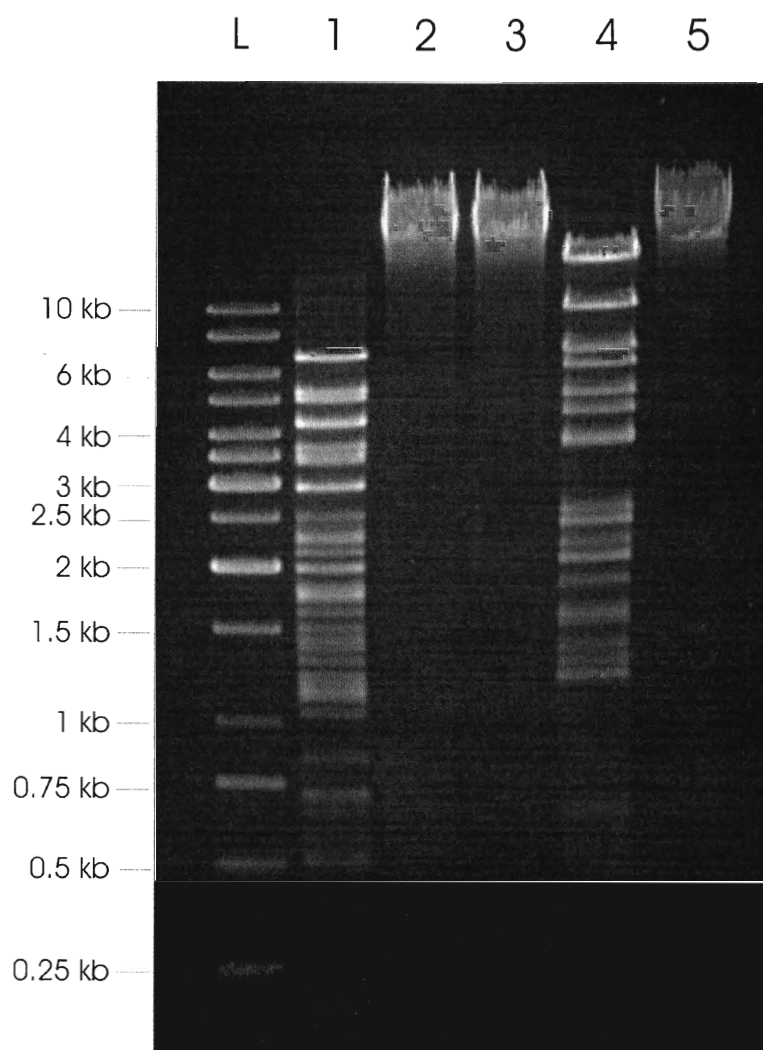


Figure 3-2. RFLP patterns resulting from the digestion of genomic  $\phi$ Ea21-4 DNA each of *MvnI* (lane 1), *BglII* (lane 2), *BamHI* (lane 3), and *EcoRI* (lane 4). Undigested genomic DNA was loaded in lane 5.

```

      10      20      30      40      50      60
φEa1 lys  MSVKKALAGGACSLALVTASFFGIVTDKVRISQEGLEHLIDCEGCKRQAYKDGAGVPTAG
      ::                               ::: . :  :: . .:: : ::::: :
φEa21-4 endo  MEV-----SQKGQQALEVMEGFSAKAYLDVAGVPTIG
                        10      20      30

      70      80      90      100      110
φEa1 lys  VGSTI----GIVMGRLYTDGEVAKM-LAKDVMI----AEQCLNRNVKVDLNQGEWDAYVS
      ::      . ::  :  ::::  :: ::      . . : . ::  . . . . : :
φEa21-4 endo  FGDTSVRARKVKMGDT-TTLEAAKAELALDLHDFKSGVEKYLAKAVK-GTTQNQFDALVI
                        40      50      60      70      80      90

      120      130      140      150      160      170
φEa1 lys  FVFNVGCSAFVSSTTYRILNGVKPGTRIQACEAMGMWNKITVNGVKVFSQGVYNRRIKDM
      ::::: . : . . . :  : . :  :  . . . . . : : : . . : : : . .
φEa21-4 endo  FAYNVGLTNFASSSVLR--NHLA-GDFEAAAKSFALWNKITVKGKKVVSGLVNNRAKEI
                        100      110      120      130      140

φEa1 lys  ALCVKY---M
      . . .
φEa21-4 endo  EIYLSHNYGV
      150

```

Figure 3-3. Similarity between φEa21-4 endolysin and the φEa1 lysozyme. (32% identity)

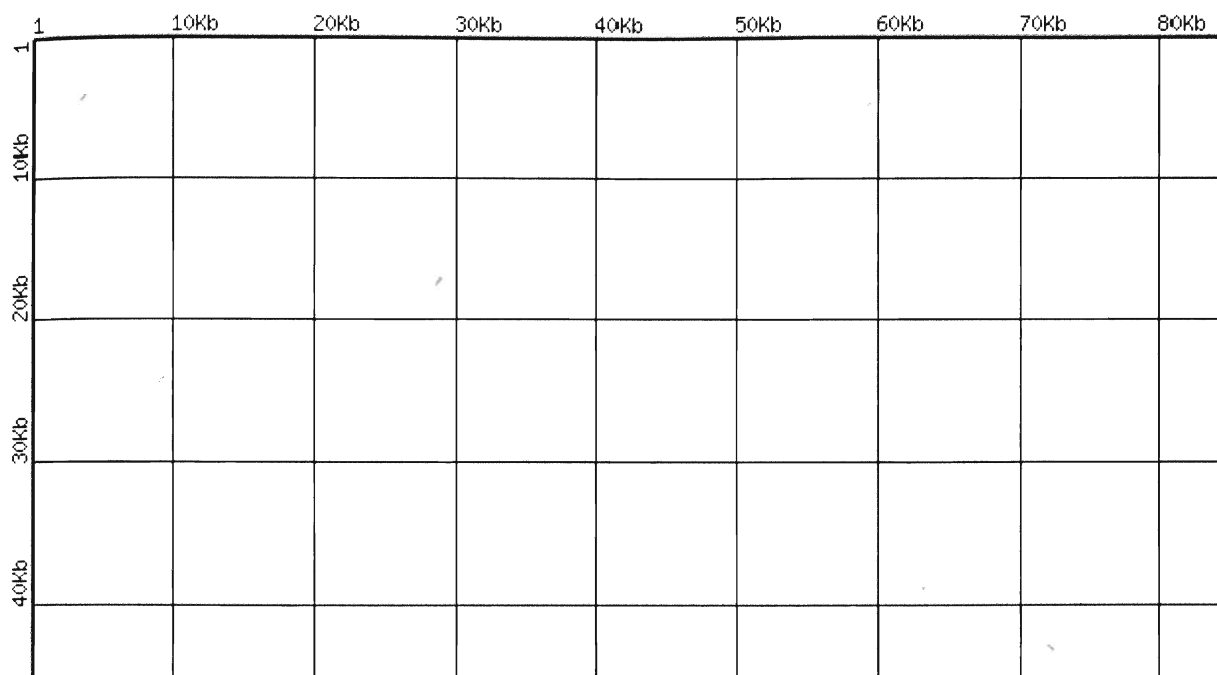


Figure 3-4. Genome-wide similarity between the  $\phi$ Ea21-4 genome (horizontal axis) and the  $\phi$ Era103 (vertical axis) genome. The alignment was constructed using the tblastx engine with a sliding window of 50 bp. Regions with at least 50% identity are indicated in red, and regions with at least 20% identity are indicated in orange.



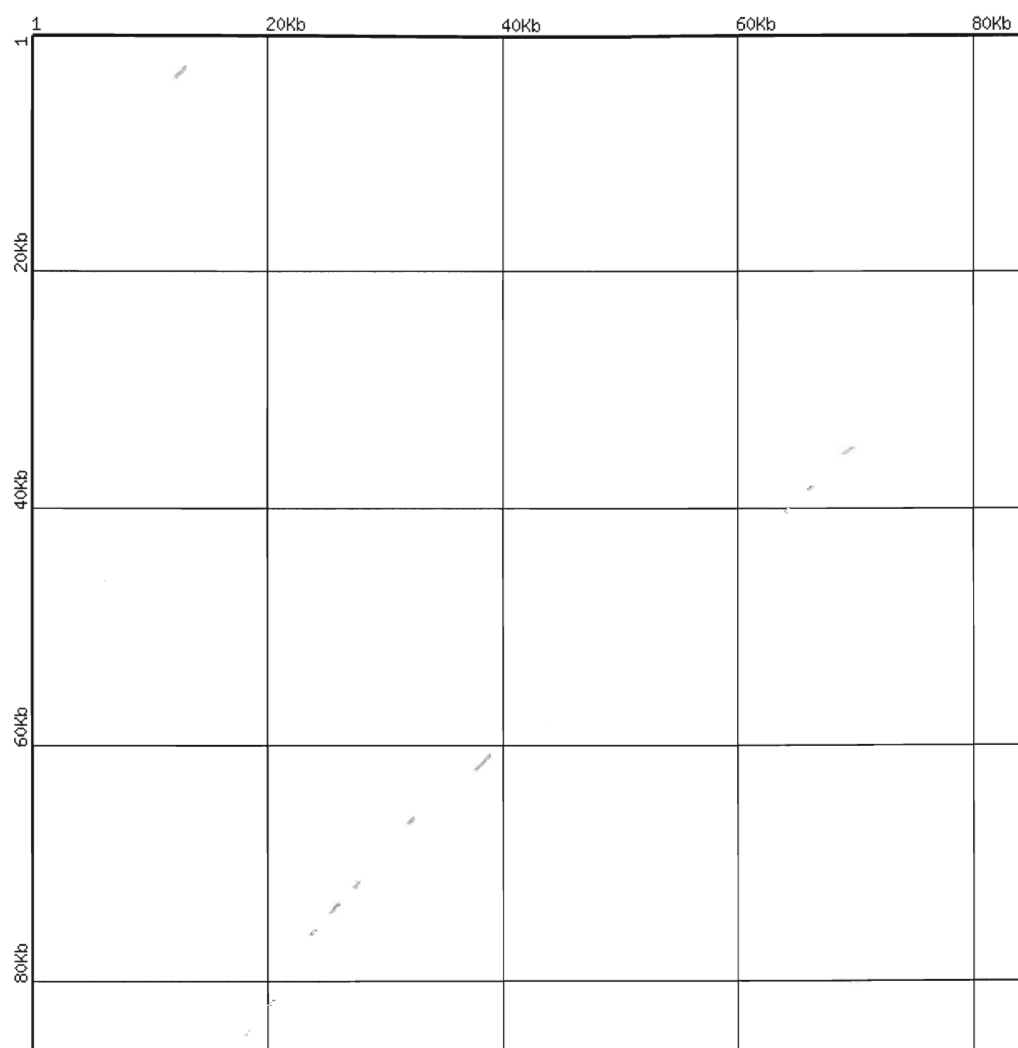


Figure 3-5. Genome-wide similarity between the  $\phi$ Ea21-4 genome (horizontal axis) and the *Salmonella* phage Felix genome (vertical axis). The alignment was constructed using the tblastx engine with a sliding window of 100 bp. Regions with at least 65% identity are indicated in red, and regions with at least 40% identity are indicated in orange.

Table 3-3 summarizes the general features of the  $\phi$ Ea21-4 genome, compared to the two other *E. amylovora* phage genomes from which sequence information is available, and to the Felix genome. The single-copy genome of  $\phi$ Ea21-4 is 84,676 bp with a GC content of 43.8%.

The  $\phi$ Ea21-4 genome is similar in size to the Felix genome, and about twice the size of the two *E. amylovora Podoviridae* genomes. Both the  $\phi$ Ea1 and Era103 genomes are only slightly less GC-rich than *E. amylovora*, whereas the  $\phi$ Ea21-4 and Felix genomes are substantially less GC-rich than their respective host genera.

### *Gene Annotation*

A total of 82 ORFs were predicted by both the GeneMark.hmm and Fickett's testcode algorithms (Table 3-4). Since all ORFs in a phage genome are predicted to be translated, functional predictions were based on similarities between the translated sequence of each predicted gene, and all possible translations of the global nucleotide database. There were no significant similarities found between 23% of the translated ORFs and any previously described gene sequences. These ORFs were classified as "predicted proteins". For 16% of the predicted ORFs, there was not enough similarity to described sequences to assign a putative function, but there were regions within the ORF that were sufficiently similar to described functional domains or phage-related proteins to support their identification as a gene. A further 29% of the predicted ORFs showed no significant similarity to any sequences except a predicted ORF in the *Salmonella* phage Felix genome. These ORFs, for which at least one significant match was obtained, were classified as unknown. The remaining 31% of the predicted ORFs were assigned a putative identification based on similarities to known proteins.

Table 3-3. General features of the  $\phi$ Ea21-4 genome compared to two other *E. amylovora* phages,  $\phi$ Ea1(h) and Era103, and *Salmonella* phage, Felix.

Feature	$\phi$ Ea21-4	$\phi$ Ea1(h) <sup>a</sup>	Era103	Felix
Size	84.7 kb	approx. 37 kb <sup>b</sup>	45.4 kb	86.2 kb
G+C content	43.8%	50.4%	49.8%	39.0%
G+C content of host Genus	50-54% <sup>c</sup>	50-54%	50-54%	50-53% <sup>d</sup>
Total number of predicted ORFs	82		53	
Average ORF length	800 bp		785 bp	
Percentage of genome constituting coding regions	78%		91.5%	91%
Morphology	<i>Myoviridae</i>	<i>Podoviridae</i> <sup>e</sup>	<i>Podoviridae</i> <sup>f</sup>	<i>Myoviridae</i> <sup>g</sup>

<sup>a</sup> based on published 3.3 kb sequence only (Kim, Salm, & Geider, 2004)

<sup>b</sup> estimate based on RFLP (Gill, 2000)

<sup>c</sup> (Hauben & Swings, 2001)

<sup>d</sup> (Le Minor, 1984)

<sup>e</sup> (Ritchie & Klos, 1979)

<sup>f</sup> probable morphology, based on Vandenberg et al (1985), and presence of depolymerase

<sup>g</sup> (Lindberg & Holme, 1969)

Table 3-4. Predicted genes of  $\phi$ Ea21-4.

ORF	Start	End	Protein Size (aa)	Putative Function or Conserved Domain	Selected best tblastn matches <sup>a</sup>	%Identity (%Similarity) / # residues
79	4	201	66	Predicted protein	NS <sup>b</sup>	
80	269	505	79	Predicted protein	NS	
81	577	825	83	Predicted protein	NS	
82	2461	2273 *	63	Predicted protein	NS	
1	2694	2464 *	77	Predicted protein	NS	
2	3657	3184 *	157	Endolysin	Felix lysis prot. (AF320576),	44 (62) / 156
				Pfam: phage lysozyme/ O-glycosyl hydrolase domain (residues 28-145)	phage Gifsy-2 lysozyme (NP_460003)	40 (54) / 130
3	4321	3707 *	204	Structural (21kDa);	Ig-like fold domains of bacterial proteins	
				Pfam: Big-2 family Ig-like domain (residues 123-301)		
4	4942	4325 *	205	Unknown	Hyp. Felix prot. (AAQ14772)	28 (45) / 205
5	5738	6103	121	Unknown	Hyp. Felix prot. (AAQ14773)	58 (77) / 118
6	9703	10437	244	$\lambda$ Red-like protein	Hyp. Felix prot (AAQ14777)	43 (67) / 240
				Pfam-B: Red-like domain families 66931 (residues: 3-149) and 94232 (residues: 194-242)		
7	10963	12567	534	Packaging terminase, large subunit	Hyp. prots: Felix (AAQ14779),	69 (82) / 532
				Pfam: Terminase-6 domain (residues: 90-505)	<i>Mesorhizobium loti</i> (BAB53654), phage psiM2 terminase large subunit (NP_046964)	40 (57) / 445
8	12573	14021	482	Unknown	Hyp. prots: Felix (AAQ14780),	66 (82) / 482
					<i>Corynebacterium efficiens</i> (BAC17652)	25 (45) / 263
9	14028	14522	164	Unknown	Hyp. Felix prot (AAQ14781)	34 (58) / 165

ORF	Start	End	Protein Size (aa)	Putative Function or Conserved Domain	Selected best tblastn matches <sup>a</sup>	% Identity/ % Similarity (# residues)
10	14522	14854	110	Unknown	Hyp. Felix prot. (AAQ14782)	43 (71) / 107
11	14862	16232	456	Prohead protease  Pfam: peptidase S49 family (residues: 133-285) Pfam-B: family 3464 of capsid proteases (residues31-130)	Hyp. Felix prot. (AAQ14669), phage BcepNazgul ClpP protease (NP_918994)	47 (64) / 429  33 (56) / 298
12	16244	16615	123	Structural (11 kDa)	Hyp. Felix prot. (AAQ14784)	42 (58) / 124
13	18232	18717	161	Unknown	Hyp. prots: Felix (AAQ14787), Vibrio cholerae (ZP_00755065)	52 (72) / 161 30 (48) / 143
14	18714	19100	128	Unknown	Hyp. Felix (AAC24149)	54 (70) / 131
15	19100	19708	202	Unknown	Hyp. Felix (AAQ14789)	47 (64) / 192
16	19713	21071	452	Structural (47 kDa)  Pfam-B: phage-related family 12740 (residues 190-397)	Hyp. prots: Felix (AAQ14790), <i>H. Influenzae</i> (AAX88346)	59 (75) / 450 30 (48) / 280
17	21087	21542	151	Structural (14 kDa)  Pfam-B: phage-related family 28462 (residues: 7-140)	Hyp. prots: Felix (AAQ14791), <i>H. Influenzae</i> (AAT40857)	83 (89) / 148 28 (52) / 125
18	21600	22010	136	Unknown	Unk. Felix (AAQ14792)	36 (61) / 132
19	22247	24469	740	Possible tail tape measure  Pfam: DNA-binding family 130203 (residues 1-306), phage tail tape measure family 2595 (residues 362-404), membrane protein family 117417 (residues 421-551)	Unk. Felix (AAQ14794) phage tail tape measure prots: BcepNazgul (NP_918983), B3 (AAQ13967)	39 (57) / 751  23 (40) / 412 28 (45) / 283

ORF	Start	End	Protein Size (aa)	Putative Function or Conserved Domain	Selected best tblastn matches <sup>a</sup>	% Identity/ % Similarity (# residues)
20	24473	25300	275	Unknown	Hyp. prots: Felix (AAQ14795) <i>Erwinia carotovora</i> (CAG75399)	39 (55) / 246 35 (50) / 251
21	25300	25623	107	Unknown	Hyp. Felix (AAQ14796)	55 (75) / 106
22	25633	26718	361	Unknown	Hyp. prots: Felix (AAQ14797), <i>Haemophilus influenzae</i> (AAX88354)	50 (69) / 355 22 (45) / 185
23	26718	27362	214	Baseplate assembly  Pfam: family 16888, phage baseplate-related prots	Hyp. Felix prot. (AAQ14798), P2 baseplate assembly (ZP_00708498)	48 (68) / 211 32 (47) / 192
24	27362	27778	138	Unknown  Pfam: family 18442 of phage-related proteins	Hyp. prots: Felix (AAQ14799), <i>Burkholderia</i> sp. (ZP_00426838; ZP_00986347), <i>S. enterica</i> phage-related (NP_455547)	54 (72) / 140 37 (55) / 85 32 (53) / 83
25	27778	29262	494	Baseplate component  Pfam-B: family 5270, phage P2 baseplate J-like prot.	Hyp. prots: Felix (AAQ14800) <i>Haemophilus influenzae</i> (AAX88357)	51 (67) / 486 26 (46) / 436
26	29272	30120	282	Unknown	Hyp. Felix (AAQ14801)	39 (62) / 278
27	30123	30476	117	Unknown	Hyp. Felix prot (AAQ14802)	52 (65) / 110
28	30480	31553	357	Tail fibre  Pfam: contains five repeat units common to phage tail fibres (residues: i) 227-240, ii) 241-254, iii) 255-268, iv) 293-306, v) 307-320	put. tail fibre: Felix (AAQ14803)	44 (58) / 395

ORF	Start	End	Protein Size (aa)	Putative Function or Conserved Domain	Selected best tblastn matches <sup>a</sup>	% Identity/ % Similarity (# residues)
29	31641	33149	502	Possibly structural (54 kDa)	<i>Chlorobium phaeobacteroides</i> putative Ig (ZP_00533375)	28 (44) / 173
30	33159	33893	244	Predicted protein	NS	
31	33893	37366	1157	Structural, possible tail fibre Pfam-B: undescribed 114474 family (residues 231-372)	Hyp. <i>Zymomonas mobilis</i> (AAV89024) Unk. $\phi$ KO2 (AAR83037) phage OP1 put. tail fibre component (YP_453579)	21 (36) / 733 30 (48) / 181 29 (48) / 164
32	37686	38021	111	Unknown	Unk. Felix prot. (AAQ14806)	25 (50) / 108
33	38997	38061 *	311	Thymidylate synthase	phage dTMP synthase Felix (AAQ14807) T5 (AAU05229)	43 (59) / 314 46 (59) / 265
34	39554	38994 *	186	Dihydrofolate reductase	phage DHFRs: Felix (AAQ14808), Aeh1 (AAQ17977)	36 (55) / 80 28 (51) / 152
35	40102	39554 *	182	Predicted protein	NS	
36	41198	40095 *	367	DNA ligase Pfam: ATP-dependent DNA ligase domain (residues 92-282)	Unk. Felix prot. (AAQ14813), phage Xp15 DNA ligase (YP_239300)	63 (74) / 367 24 (41) / 374
37	42715	41447 *	422	Nucleic acid-independent RNA polymerase Pfam: 1743 family (residues 3-127)	Polynucleotide adenylyltransferase: <i>Nitrosococcus oceani</i> (ABA56915), Poly-A polymerase: <i>Yersinia</i> sp. (ZP_00829667)	36 (48) / 419 35 (51) / 415
38	43073	42723 *	116	Unknown	Unk. Felix prot. (AAQ14810)	36 (52) / 119

ORF	Start	End	Protein Size (aa)	Putative Function or Conserved Domain	Selected best tblastn matches <sup>a</sup>	% Identity/ % Similarity (# residues)
39	44058	43180 *	292	Unknown  Pfam: Band_7 integral membrane proteins, inc HflK & HflC-like (residues 23-239)	put. serine protease: phage P27 (CAC83523), <i>Shigella flexneri</i> (AE014073)	37 (58) / 256 37 (58) / 265
40	44985	44668 *	105	Predicted protein	NS	
41	48525	45835 *	896	DNA polymerase	DNA polymerase: Felix (AAQ14817), PaP3 (AAL85539)	62 (74) / 894 24 (43) / 413
42	49040	48564 *	158	H-N-H endonuclease  PfamB-25515	phage endonuclease: RB16 (AAY44388), Xp10 (AAP58717)	43 (60) / 139 41 (57) / 140
43	49278	49685	135	Unknown	Hyp. Felix (AAQ14818)	49 (70) / 131
44	49729	50292	187	Predicted protein	NS	
45	50285	51136	283	Predicted protein	NS	
46	51196	52182	328	Deoxyribonucleoside monophosphate kinase	dNMP kinase: Felix (AAQ14820), KVP40 (NP_899571)	31 (46) / 235 30 (46) / 220
47	52175	54088	637	DNA primase-helicase	primase/helicase: Felix (AQ14821) $\phi$ gh-1 (AAO73154)	70 (83) / 609 27 (45) / 590
48	54191	55054	287	Unknown	Unk. Felix prot. (AAQ14822)	42 (58) / 267
49	55078	56196	372	DNA-directed DNA polymerase  Pfam: 5' - 3' exodeoxyribonuclease (residues 268-360)	Felix (AAQ14823) <i>Thermus</i> sp. polA (CAA46900)	53 (69) / 347 28 (42) / 194
50	56189	56704	171	Unknown	Hyp. phage prots: Felix (AAQ14824), Cyanophage P-SSM2 (YP_214449)	60 (77) / 156 32 (52) / 111
51	57000	57746	248	Unknown  Pfam: glutaredoxin family (residues 162-205)	Unk. Felix prot. (AAQ14826)	37 (57) / 245



ORF	Start	End	Protein Size (aa)	Putative Function or Conserved Domain	Selected best tblastn matches <sup>a</sup>	% Identity/ % Similarity (# residues)
52	58405	60639	744	ribonucleotide triphosphate reductase ( $\alpha$ )	RN TPRase ( $\alpha$ ): Felix (AAQ14827), $\phi$ KZ (NP_803872)	57 (75) / 746 39 (57) / 738
53	60694	61791	365	ribonucleotide triphosphate reductase ( $\beta$ )	RN TPRase ( $\beta$ ): Felix (AAQ14828), $\phi$ KZ (NP_803871)	50 (68) / 348 31 (54) / 369
54	62718	63827	369	Predicted protein	NS	
55	64309	64821	170	Unknown	Unk. Felix prot (AAQ14737)	44 (62) / 156
				Pfam-B: undescribed family 115568 (residues 46-165)		
56	65094	65939	281	Phosphoribosyl pyrophosphate synthetase	Unk. Felix prot. (AAQ14838) <i>Cytophaga hutchinsonii</i> PRPP synthetase (ZP_00310994)	40 (59) / 249 32 (54) / 198
				Pfam: Phosphoribosyl-pyrophosphate kinase domain (residues 142-250)	Unk. phage K, G1prot	25 (43) / 201
57	65953	67782	609	NMN phosphoribosyl transferase	Hyp. Felix prot. AAQ14840) <i>C. violaceum</i> Na PRTase (NP_899704)	46 (61) / 591 38 (51) / 593
				Pfam: nicotinamide phosphoribosyl-transferase domain (residues 185-574)	phage P100 (YP_406440)	35 (53) / 634
58	67942	68355	137	Unknown	Hyp. <i>Reinekea</i> sp. Prot. (ZP_01114443), <i>Pseudoalteromonas tunicata</i> putative solute/DNA competence effector (ZP_01134797)	27 (48) / 95 29 (52) / 101
59	68358	68684	108	Predicted protein	NS	
60	68934	71384	816	rIIA	rIIA from phages: Felix (AAQ14746), RB43 (YP_238977)	33 (49) / 787 38 (53) / 296

ORF	Start	End	Protein Size (aa)	Putative Function or Conserved Domain	Selected best tblastn matches <sup>a</sup>	% Identity/ % Similarity (# residues)
61	71436	72563	375	rIIB	rIIB from phages: Felix (AAQ1474), RB69 (NP_861963), T4 (NP_049889)	57 (72) / 376 43 (60) / 230 41 (61) / 240
62	72889	73452	187	Unknown  Pfam-B: NrdA-like protein family	Hyp. <i>Streptomyces</i> (NP_828254) and <i>Mesorhizobium</i> (EAN04910) prots. <i>Burkholderia</i> phage T4 NrdA-like prot. (NP_919003)	35 (49) / 103 40 (51) / 82 34 (43) / 79
63	73693	74205	170	Unknown  Pfam-B: undescribed family 14879 from certain <i>Myoviridae</i> and $\epsilon$ proteobacteria (residues 20-164) Pfam-A: low significance match to SLT transglycosylase family	Unknown Felix prot. (AAQ14595) Conserved hyp. prot. in <i>Aeromonas</i> phages Aeh1 (AAQ17934) 31 (YP_238834), <i>Enterobacteria</i> phages 44RR2 (AAQ81426), RB49 (AAL15146)	45 (59) / 169 31 (51) / 170 32 (47) / 184 32 (47) / 184 39 (53) / 130
64	74207	74857	216	Unknown	Unknown Felix prot. (AAQ14597)  Enterobacteria phage RB69 (NP_861919)	34 (55) / 149 25 (46) / 149
65	74940	75407	155	Unknown	Unknown Felix prot. (AAQ14749)	63 (75) / 120
66	75937	76302	121	Unknown	Hypothetical Felix prot. (AAQ14765)	27 (48) / 120
67	76304	76870	188	Predicted protein	NS	
68	76912	77268	118	Unknown	Hypothetical <i>E.coli</i> (ZP_00730579)  Hypothetical Felix (AQ14750)	34 (53) / 82 30 (50) / 86
69	77415	78197	260	Unknown  Pfam: A1pp ADP-ribose binding domain (residue 30-156)	Unknown Felix prot. (AAQ14754)  <i>Acinetobacter</i> phage 133 (AAT38492)	44 (60) / 153 38 (49) / 154
70	78657	79004	115	Predicted protein	NS	

ORF	Start	End	Protein Size (aa)	Putative Function or Conserved Domain	Selected best tblastn matches <sup>a</sup>	% Identity/ % Similarity (# residues)
71	79043	79708	221	Predicted protein	NS	
72	79798	80295	165	Unknown	Unknown Felix prot. (AAQ14755)	46 (65)/166
73	80734	81150	138	Predicted protein	NS	
74	81399	81734	111	Predicted protein	NS	
75	82195	82782	195	Predicted protein	NS	
76	82861	83184	108	Predicted protein	NS	
77	83576	83851	92	Unknown	Unknown Felix prot. (AAQ14618)	47 (57) / 88
78	84208	23	164	Unknown	Unknown Felix prot. (AAQ14759)	32 (49) / 126

\* ORFs marked with an asterisk are transcribed from the antiparallel strand

<sup>a</sup> The listed hits represent the best local alignment, and the most informative of the next best local alignments. These are not necessarily the most closely related sequences. In most cases these alignments have an expect value greater than  $e^{-4}$ , though in cases where the only match was a hypothetical Felix protein, the expect value was between 0.01 and  $e^{-4}$ .

<sup>b</sup> "NS" indicates that no significant local alignment was found between any portion of the indicated ORF and any portion of sequences

A single cluster of 19 tRNA genes was predicted in the  $\phi$ Ea21-4 genome. Table 3-5 summarizes these predictions. The predicted secondary structure of each putative tRNA gene is consistent with a functional tRNA. This cluster lies between orf-5 and orf-6.

### *Structural Proteins*

Phage genomes include a limited number of genes encoding the structural proteins that make up the free virion. These genes were identified by denaturing purified  $\phi$ Ea21-4 virions, separating the component proteins, and matching the N-terminal sequence of each to the predicted protein sequences in the  $\phi$ Ea21-4 genome. Figure 3-6 shows the outcome of electrophoretic separation of the phage structural proteins. Ten bands were visible to the naked eye after about 1 h of destaining, though not all of these are visible in the gel photo. All of these proteins are listed in Table 3-6.

There was an insufficient quantity of the 23 kDa, 54 kDa, and 120 kDa proteins for N-terminal sequencing, and the 36 kDa protein appeared to be N-terminally blocked, since no sequence was obtained from more than 32 ng of protein. Partial sequence was obtained from the 38 kDa protein, but the encoding orf could not be identified. The four proteins for which a clean sequence was produced could all be matched to predicted ORFs in one region of the genome. None of these ORFs had been identified based on sequence similarities, but all were significantly similar to predicted proteins in the Felix genome.

The 42 kDa band contained two proteins of similar size, so a sequence could not be determined for either. An attempt was made to resolve these proteins by isoelectric focusing, but no protein bands were visible following gel electrophoresis of the concentrated fractions.

Table3-5. Locations and features of tRNAs encoded by the  $\phi$ Ea21-4 genome.

tRNA Type	Anti-codon	tRNA start	tRNA end
Pro	TGC	6394	6471
Met	CAT	6847	6921
Tyr	GTA	7012	7094
Met	CAT	7101	7174
Asp	GTC	7254	7330
Lys	TTT	7732	7807
Lys	CTT	7815	7891
Met	CAT	7903	7978
Ile	GAT	7990	8064
Gln	TTG	8071	8146
Arg	TCT	8153	8229
Leu	CAA	8647	8723
Trp	CCA	8935	9010
Val	TAC	9102	9177
Leu	AAG	9184	9261
Pseudo-tRNA	ACG	9268	9344
Gln	CTG	9347	9423
Undetermined		9430	9506
His	GTG	9510	9586

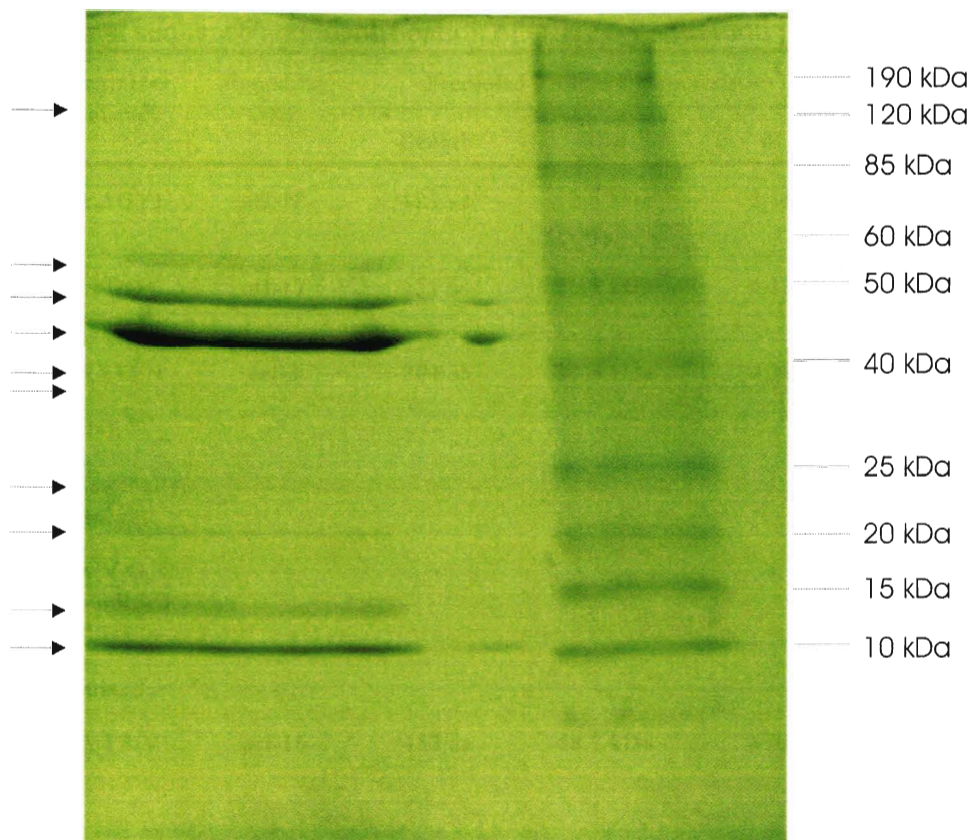


Figure 3-6. Electrophoretic separation of  $\phi$ Ea21-4 structural proteins. Apparent sizes of the pre-stained ladder are indicated on the right. Arrows on the left point to the locations of each of the visible phage structural proteins.

Table 3-6. N-terminal sequence and identification of  $\phi$ Ea21-4 structural proteins.

Approximate Size (kDa) <sup>a</sup>	N-terminal Sequence	Encoding ORF	Predicted Protein Characteristics <sup>b</sup>			analogous Felix gene <sup>c</sup> (Accession No.)
			Length	Size	pI	
11	AYAGYI	orf-12	123 aa	12.8 kDa	5.18	orf-135 (AAQ14784)
14	SLFQQY	orf-17	151 aa	16.3 kDa	4.19	orf-155 (AAQ14791)
21	ARETFN	orf-3	204 aa	21.9 kDa	4.99	orf-79 (NP_944848)
23	NE <sup>d</sup>	- <sup>e</sup>	-	-	-	-
36	N-terminally blocked	-	-	-	-	-
38	H/A/V,G, C or modified, LNL	-	-	-	-	-
42	mixed	-	-	-	-	-
47	AYTPIV	orf-16	452 aa	48.7 kDa	4.79	orf-150 (AAQ14790)
54	NE	-	-	-	-	-
120	NE	-	-	-	-	-

<sup>a</sup> As observed on SDS-PAGE, estimated from stained ladder<sup>b</sup> As predicted by Clone Manager (SciEd Central), from the sequence data<sup>c</sup> Based on Bacteriophage Felix complete genome sequence: NC\_005282.1<sup>d</sup> "NE" indicates that insufficient protein was extracted from the PVDF blot to allow N-terminal sequencing;<sup>e</sup> "-" indicates information that is unknown as a result.

## Discussion

The sequencing process revealed several characteristics of the  $\phi$ Ea21-4 genome. More than half of the genome was not represented in the random shotgun library, despite the presence of terminator sequences designed to prevent transcription of genes cloned into the vector. At least one of the unrepresented regions contained the endolysin gene, which is to be expected since this gene product is toxic to bacterial cells. For the same reason, the holin gene almost certainly lies in one of the regions that was not contained in the shotgun library. However, it is not known why so much of the genome was not represented. By comparison, the authors responsible for sequencing the genome of the KVP40 vibriophage reported that 24 regions comprising only 25% of that 245 kb genome were not represented in the shotgun library (Miller et al., 2003a). The use of insert sizes smaller than the 2-4 kb fragments used in these two studies would be more likely to produce shotgun libraries with greater genome coverage.

Information about the packaged form of the genome can also be inferred from the sequencing process. The replication strategy of linear double-stranded DNA phages is related to the form of the packaged virion DNA, in that there must be a means of preserving the genetic information upstream of the initial replication primers (Jardine et al., 2006). Phages such as lambda have 5' cohesive ends that permit circularization of the phage genome upon infection. The replication of the  $\phi$ 29 genome is primed by glycoproteins that are covalently linked to the 5' termini. The T3 and T7 phages have terminal direct repeats that allow recombination. Each of these replication strategies results in packaged genomes with a defined unit length and identical terminal sequences for all virions in the population. Alternatively, the genomes of a population may also be circularly permuted with respect to each other, as seen in T4, SPP1, and P22. This is



the result of less specific terminal redundancy, in which phage genomes are replicated as a concatemer, and a length of DNA greater than 100% of the single-copy genome is packaged into each phage head (termed “headful packaging”). Direct, custom-primed sequencing from the termini of the final contig was halted when the same 182 bp sequence was obtained from both ends.  $\phi$ Ea21-4 clearly uses direct terminal repeats to prevent sequence loss during replication, but these repeats could be defined, as in T7, or circularly permuted, as in T4-like phages.

The 82 ORFs listed in Table 3-4 are almost certainly an underestimate of the total number of  $\phi$ Ea21-4 genes. Most phage genomes contain very densely packed genes, yet these 82 ORFs comprise only 78% of the single-copy genome. In comparison to some other phages of the Enterobacteriaceae, coding sequences comprise 91.5% of the Era103 genome, 92.1% of the KVP40 genome, 93% of the T4 genome, and at least 87% of the lambda genome (Summer et al., 2007; Miller et al., 2003a; Miller et al., 2003b; Sanger et al., 1982). There are four places in the  $\phi$ Ea21-4 genome where two adjacent ORFs are separated by more than 1000 bp: between orf-81 and orf-82, between orf-4 and orf-5, between orf-5 and orf-6, and between orf-12 and orf-13. The region between orf-5 and orf-6 encodes a tRNA cluster. The other three regions are excellent candidates to harbour ORFs that were not identified by the conservative methods employed here.

Nineteen of the predicted ORFs, or 23%, are completely unique to  $\phi$ Ea21-4, containing no recognizable conserved domains and having no significant similarity to the products of known or predicted genes. An additional 34 ORFs, or 42% appear to be conserved hypothetical proteins, with similarities to unidentified predicted gene products in at least one other phage genome. The discovery of unique genes is a common theme in phage genome sequencing (Kapfhammer et al., 2002; Miller et al., 2003a; Pedulla et al., 2003; Rohwer, 2003), and is indicative of a substantial

pool of information about phage ecology that has yet to be tapped.

Nineteen phage-encoded tRNAs were predicted, including one pseudo-tRNA. Of the 18 predicted to be functional, one is suspicious since the anti-codon could not be predicted. The purpose of phage-encoded tRNAs can be to facilitate the incorporation of those amino acids for which the phage and host exhibit substantially different codon usage patterns. This appears to be the case for a cluster of eight tRNAs in T4 (Kunisawa, 1992). However, the vibriophage KVP40 genome contains a cluster of 25 apparently functional tRNAs, and yet significant codon usage differences between host and phage were not observed for many of the corresponding amino acids (Miller et al., 2003a). The authors suggest that there may be different codon usage patterns within KVP40 gene clusters, rather than a genome-wide trend. Both possibilities should be considered for  $\phi$ Ea21-4.

Few protein-encoding genes involved in transcription or translation were identified in the  $\phi$ Ea21-4 genome. Orf-37 encodes a nucleic acid-independent RNA polymerase. This protein family includes the poly(A)-polymerase enzymes that are responsible for generating the poly(A) tail of nascent mRNAs, as well as the tRNA nucleotidyltransferases that attach the CCA triplet to the 3' end of tRNA. The latter function would be consistent with the presence of phage-encoded tRNA genes, two of which are not predicted to have genomically encoded 3' CCA termini.

The  $\phi$ Ea21-4 genome includes genes for many aspects of nucleic acid metabolism. Dihydrofolate reductase and thymidylate synthase both participate in the cycling of 5,6,7,8-tetrahydrofolate to produce deoxythymidine phosphate. The two proteins are encoded by adjacent ORFs, and are likely transcribed as part of a single operon since the genes are separated by 3 nucleotides. The ribonucleotide triphosphate reductase holoenzyme encoded by orf-52 and orf-53

is responsible for the conversion of ribonucleotides into their deoxyribonucleotide forms. The product of orf-51, which immediately precedes these two genes, contains a glutaredoxin domain, and may therefore function as an electron carrier for ribonucleotide reductase holoenzyme. Deoxyribonucleoside monophosphate kinase also plays a role in nucleotide turnover, catalyzing the phosphorylation of dNMP, at the expense of ATP.

$\phi$ Ea21-4 also appears to encode a NadV homolog. NadV is a principle component of a pyridine nucleotide salvage pathway that has been identified in a few bacterial genomes and, recently, in vibriophage KVP40 (Martin, Shea, & Mulks, 2001; Miller et al., 2003a). The NadV protein, a nicotinamide phosphoribosyltransferase, converts nicotinamide to nicotinamide mononucleotide. The KVP40 genome also appears to encode proteins that catalyze the active uptake of nicotinamide mononucleotide, its conversion to  $\text{NAD}^+$ , and its regeneration from  $\text{NAD}^+$ . Homologues of these other genes are not found in  $\phi$ Ea21-4, none of the described genes are found in *E. amylovora*. Interestingly, *E. amylovora* displays a strict requirement for nicotinic acid (Hauben & Swings, 2005), which suggests that the  $\phi$ Ea21-4-encoded NadV may play a role in phage adaptation to or interference with host cell metabolism.

It is not surprising that the small number of genes identified by sequence similarity alone included those for thymidylate synthase, DNA polymerase, ribonucleotide reductases, and dihydrofolate reductase. Families of these genes appear to have diverged prior to the divergence of eukaryotes and prokaryotes, and therefore members of one family tend to be similar across different branches of the phylogenetic tree, with less similarity between different families in closely related organisms (Brüssow & Kutter, 2005).

*Escherichia coli* phage T4, the best characterized of the *Myoviridae*, consists of 35

different structural proteins (Mosig & Eiserling, 2006). The  $\phi$ Ea21-4 genome contains genes for at least 10, including the four that were identified by N-terminal sequencing. Orf-3 encodes a 21 kDa structural protein that has an Ig-like domain from the Big-2 family (aa region). The immunoglobulin fold is very common and very widely dispersed (Halaby, Poupon, & Mornon, 1999). The Big-2 type consists of seven  $\beta$ -strands arranged in two parallel sheets, and is most commonly found in proteins involved in bacterial cell adhesion (Fraser et al., 2006). At least one protein with an Ig-like domain is found in the proteome of about 25% of fully sequenced *Caudovirales* phages, usually within tail fiber, baseplate wedge initiator, major tail, major head, and highly immunogenic outer capsid (HOC) proteins (Fraser et al., 2006). In this case, orf-3 is more likely to be one of the head proteins, since the genes for tail components and tail assembly seem to be clustered on the other side of a putative prohead protease, in the vicinity of orf-31 through orf-19. The predicted orf-3 gene product did align with part of a Felix protein annotated as “HK97 major tail protein”. This Felix protein contains an Ig-like domain from the I-set family, and its annotation has been questioned because it is based on similarity to a *Siphoviridae* tail protein (Felix belongs to *Myoviridae*) and thus probably reflects the similarity of their Ig-like domains rather than true functional homology of the whole proteins (Fraser et al., 2006). Similarly, the alignment between these  $\phi$ Ea21-4 and Felix gene products is almost certainly based on the common features of the Big-2 and I-set Ig-like domains.

Orf-12 encodes an 11 kDa structural protein that shares 40% identity with a predicted Felix protein of unknown function. Since an adjacent gene, orf-11, has a conserved ClpP serine protease domain, which is common to prohead protease enzymes, it is possible that orf-12 encodes one of the capsid components. Orf-16 and orf-17 encode 47 kDa and 21 kDa structural

proteins that fall between the region associated with head morphogenesis and the region associated with tail morphogenesis. The proteins encoded by orf-16 and orf-17 are 58% and 82% identical to adjacent predicted Felix proteins of unknown function. The ~120 kDa structural protein seen on the SDS-PAGE gel is probably encoded by orf-31, the largest gene in the  $\phi$ Ea21-4 genome. The predicted product of this gene is 1157 amino acids, has a predicted mass of 130 kDa, and it is similar to the putative tail fibre protein of *Xanthomonas oryzae* phage OP1, another tailed, dsDNA phage.

The morphogenesis of mature  $\phi$ Ea21-4 virions also involves DNA packaging. Orf-7 contains a conserved phage terminase domain. Terminase holoenzymes consist of two proteins, a large and a small subunit (Jardine & Anderson, 2006). They target the DNA to the prohead, initiate packaging, power DNA translocation by ATP hydrolysis, and terminate packaging. Assuming that orf-7 does, in fact, encode one of the terminase subunits, presumably either orf-6 or orf-8 encodes the second subunit of the holoenzyme.

The final stage of lytic phage replication is host cell lysis. As with all known double-stranded nucleic acid phages,  $\phi$ Ea21-4 appears to use a holin-endolysin lytic strategy. The  $\phi$ Ea21-4 endolysin bears little similarity to the  $\phi$ Ea1 or T4 lysozyme, and is therefore not likely to be a muramidase, or “true lysozyme”. No putative holin gene was identified in the  $\phi$ Ea21-4 genome, but this is perhaps not surprising given the incredible degree of holin diversity. There are currently more than 250 putative phage holins in over 50 families, and the lethality of the gene product has prevented functional analysis of all but a few of them (Young & Wang, 2006). The key feature is the presence of transmembrane domains, usually two or three, with cytoplasmic C-termini (classes II and I, respectively), or two transmembrane domains with a

large C-terminal periplasmic domain (class III) (Young & Wang, 2006). The holin is responsible for the precise timing of endolysin-mediated host cell lysis.

On a whole-genome scale,  $\phi$ Ea21-4 is not similar to the  $\phi$ Era103 genome or the sequenced portion of  $\phi$ Ea1. The  $\phi$ Ea1 fragment only contains three genes: a depolymerase gene, and the lysozyme and holin genes just discussed. No depolymerase gene was identified within the  $\phi$ Ea21-4 genome. Depolymerase is an virion-bound exopolysaccharide-degrading enzyme. It is most commonly isolated from lytic *Podoviridae* (Scholl & Merrill, 2005), which is consistent with its presence in  $\phi$ Ea1 and its absence in  $\phi$ Ea21-4. The morphology of  $\phi$ Era103 has not been described in detail (Vandenbergh, Wright, & Vidaver, 1985; Vandenbergh & Cole, 1986), but the production of a depolymerase and the similarity of its genome size to that of  $\phi$ Ea1 strongly suggest that it, like  $\phi$ Ea1, is a member of the *Podoviridae*.

The only significant similarity between  $\phi$ Ea21-4 and a previously described phage is with *Salmonella* phage Felix 01. Felix is a ubiquitous virulent phage that infects almost all *Salmonellae* and is therefore used as a diagnostic and typing phage (Cherry et al., 1954). Like  $\phi$ Ea21-4, Felix belongs to the *Myoviridae*. Its head has been reported to be either 72 nm (Ackermann & Nguyen, 1983) or 60 nm across (Lindberg & Holme, 1969), the latter of which is the same size as  $\phi$ Ea21-4. The Felix genome is 86.2 kb, which is only 1.5 kb larger than the  $\phi$ Ea21-4 genome, and has a lower GC content, about 39% (Sriranganathan et al., 2006). The similarity of the  $\phi$ Ea21-4 and Felix genomes appears to be largely correlated with their shared morphology. When the aligned genome sequences were compared, the regions that share 40-65% identity mostly involve the regions in  $\phi$ Ea21-4 that appear to encode tail fibre structure and assembly proteins.

There is currently no publication associated with the complete sequence of Felix, but the authors of a study dealing with an 11.5 kb fragment declared its sequence sufficiently different from known phages as to represent “the prototype of a new phage family” (Kuhn et al., 2002). *E. amylovora* phage  $\phi$ Ea21-4 would appear, then, to be the second member of this “family”. These two phages both encode 21 predicted ORFs that are not significantly similar to any known gene.  $\phi$ Ea21-4 orf-3, orf-12, orf-16, and orf-17, identified as encoding structural proteins in this study, are significantly similar to predicted Felix genes orf-79, orf-135, orf-150, and orf-155, respectively.

The organization of the  $\phi$ Ea21-4 genome is consistent with the functional clustering that is usually seen in phage genomes (Calendar, 2006). The “immediate early”, or simply “early”, genes are responsible for protecting the phage genome and mediating the transition from cellular to phage-directed metabolism. They are transcribed by host RNA polymerase, and so their promoters may resemble those of the host genome more closely than do other promoters in the phage genome. The middle genes are responsible for replicating the phage genome, and for the transcription and translation of most of the phage genome. These genes appear to encompass at least orf-33 through orf-57. Finally, the late genes are responsible for morphogenesis and lysis. (Guttman, Raya, and Kutter, 2005) Within the  $\phi$ Ea21-4 genome, the structural, assembly and lysis genes appear to encompass orf-31 through orf-2, and perhaps some of orf-70 through orf-82. No genes associated with a lysogenic life cycle were predicted.

In summary, the genome sequence of *E. amylovora* phage  $\phi$ Ea21-4 reveals this phage to be substantially different from previously characterized phages, including the extremely limited number of genetically characterized *E. amylovora* phages.

#### **Chapter 4: Real-time PCR reveals competition between *Erwinia amylovora* and *Erwinia pyrifoliae* on pear blossoms**

The following chapter is a manuscript that has been prepared for submission to Phytopathology. Submission has been delayed until licensing negotiations regarding one of the reagents are concluded.

Authorship of the manuscript is as follows: Susan M. Lehman, Won-Sik Kim, Alan J. Castle, and Antonet M. Svircev, with equal contributions by the first and second authors. First and third authors: Department of Biological Science, Brock University, 500 Glenridge Avenue, St. Catharines, ON, Canada L2S 3A1; first, second and fourth authors: Agriculture and Agri-Food Canada, Southern Crop Protection & Food Research Centre, 4902 Victoria Ave. North, P.O. Box 6000, Vineland Station, ON, Canada L0R 2E0.

Experimental contributions to this work were as follows:

Design and optimization of the described primers and probes: Dr. Kim

Specificity testing: Dr. Kim, with some contributions by S. M. Lehman

Sensitivity testing: both S. M. Lehman and Dr. Kim

Competition experiments and population monitoring: S. M. Lehman

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## Chapter 4: Real-time PCR reveals competition between *Erwinia amylovora* and *Erwinia pyrifoliae* on pear blossoms

### Abstract

*E. amylovora* and *E. pyrifoliae* are the causative agents of fire blight and Asian pear blight, respectively. The pathogens are closely related, with overlapping host ranges. Data are unavailable on the current distribution of *E. pyrifoliae*, and on the interaction between the two species when they are present together on the same host. In this study, a duplex real-time PCR protocol was developed to monitor the population dynamics of *E. amylovora* and *E. pyrifoliae* on the surface of Bartlett pear blossoms. Bacterial cells washed from blossoms were used directly as the PCR template without DNA extraction. Primers and a probe based on the *E. amylovora* levansucrase gene detected all *E. amylovora* isolates. All *E. pyrifoliae* isolates, including the Japanese *Erwinia* strains previously described as *E. amylovora*, were detected with a primer and probe combination based on the *E. pyrifoliae* *hrpW* gene. Disease appearance and severity were not significantly different in blossoms inoculated with individual *Erwinia* species or with a mixture of the two species. However, *E. amylovora* grew to higher population sizes than did *E. pyrifoliae* in both single species inoculations and in mixtures, suggesting that *E. amylovora* has a greater competitive fitness on Bartlett pear blossoms than *E. pyrifoliae*.

## Introduction

*E. amylovora* is well known as the causative agent of fire blight, a necrotic disease affecting species in the Maloidae subfamily. Fire blight was first described on apple trees in 1780 in the northeastern United States. The disease has since spread throughout much of North America, to New Zealand, England, most of Europe, and the Middle East (Bonn & van der Zwet, 2000). *E. amylovora* has a significant economic impact on commercial apple and pear crops. In the United States alone, fire blight is estimated to cost about \$100 million annually in crop loss and management costs (Norelli, Jones, & Aldwinckle, 2003). The annual disease cycle begins when *E. amylovora* present in the orchard, usually in the margins of overwintering cankers from previous year's infections, colonize open blossoms in the spring (van der Zwet & Keil, 1979). Under favourable temperature and moisture conditions, the *E. amylovora* population will grow and infect via natural openings in the blossom. Subsequent intercellular and intravascular growth of the pathogen leads to necrosis which can spread rapidly throughout the tree (van der Zwet & Keil, 1979). Chemical and biological control methods are available to prevent initial infections, but once a tree is infected, the spread of the bacteria can only be stopped by cutting out infected tissue.

*Erwinia pyrifoliae* Kim et al., was isolated in the 1990s from Asian pear trees in South Korea that showed symptoms very similar to fire blight. The isolated organism had several morphological and biochemical characteristics in common with *E. amylovora* (Rhim et al., 1999), and its 16S rRNA gene shared 99% homology with the *E. amylovora* 16S rRNA gene (Kim et al., 1999). However, DNA:DNA hybridization data and the sequences of the 16S-23S intergenic transcribed spacer regions revealed the *E. pyrifoliae* isolates to be a single species,

distinct from *E. amylovora* (Kim, Gardan, Rhim, & Geider, 1999). Additional molecular evidence for the distinction between *E. pyrifoliae* and *E. amylovora* has also been presented (McGhee et al., 2002; Maxson-Stein et al., 2003; Jock & Geider, 2004). To date, *E. pyrifoliae* has only been isolated from Asian pear trees, and has not been reported since the localized outbreaks in the 1990s (Kim et al., 2001a).

Extensive work has been done to genetically characterize *E. pyrifoliae*, but except for some small-scale pathogenicity tests on apple and pear seedlings and immature pear fruit, the *in vivo* behaviour of this organism has not been studied. Despite the genetic differences on which their classification is based, and differences in certain pathogenicity factors (Kim et al., 2002), both *E. amylovora* and *E. pyrifoliae* are pathogenic on both Asian and western pear varieties (Kim et al., 1999; Kim et al., 2001b). An understanding of how the two species interact when present together on open blossom may shed light on their current distribution, and on the potential for future spread of each organism.

Here, we describe the development of a duplex real-time PCR protocol that permits simultaneous quantification of *E. pyrifoliae* and *E. amylovora* directly from plant samples. This approach is rapid and quantitative, and thus has advantages over previously described methods for differentiating *E. amylovora* and *E. pyrifoliae* using conventional PCR and gel electrophoresis (Kim et al., 2001b). We then used this system to study the population dynamics of these two pathogens when present individually or together on the surface of Bartlett pear blossoms.

A second purpose of this study was to use the two primer and probe combinations to investigate relationships between *E. amylovora*, *E. pyrifoliae* and *Erwinia* isolates from Japan.

The latter were isolated from Asian pear trees on Hokkaido. Initial studies (Beer et al., 1996) suggested that these isolates were very similar to *E. amylovora*, which agreed with a 1981 Japanese report (cited by Beer et al., 1996). However, the subsequent identification of *E. pyrifoliae* as a distinct species from *E. amylovora*, despite many shared morphological, metabolic, and genetic similarities, prompted a reassessment of that conclusion.

## Methods

### *Bacterial Strains and Media*

Isolates of *E. amylovora*, *E. pyrifoliae*, *Pantoea agglomerans* (formerly *Erwinia herbicola*), *Erwinia carotovora*, and *Escherichia coli* were grown on Difco nutrient agar (Becton Dickinson and Company, Sparks, MD). *Pseudomonas* isolates were grown on NBY agar (Schaad, Jones, & Chun, 2001) containing 8 g/L Difco nutrient broth (BD), 2 g/L Bacto yeast extract (BD), 2.5 g/L glucose, 2 g/L K<sub>2</sub>HPO<sub>4</sub>, 0.5 g/L KH<sub>2</sub>PO<sub>4</sub>, 15 g/L Bacto agar (BD), and 1.0 mL of a sterile solution of 1.0 M MgSO<sub>4</sub>·7H<sub>2</sub>O which was added after autoclaving. All strains were incubated at 28°C, except for *E. coli*, which was incubated at 37°C. Bacteria used in these experiments are described in Table 1. All listed isolates were used for specificity testing of real-time PCR probes and primers. *E. amylovora* Ea6-4 and *E. pyrifoliae* Ep1/96 were used for competition assays.

### *Real-time PCR*

*E. amylovora* and *E. pyrifoliae* -specific primers and TaqMan probes were designed based on the levansucrase gene (GenBank: X75079) and the *hrpW* gene (GenBank: AY237642) respectively by using web-based software provided by Integrated DNA Technologies (Coralville, IA). Candidate oligonucleotide designs were analyzed to ensure compatibility in a duplex reaction. Probe and primer sequences are listed in Table 4-2. All primers and probes were synthesized by Integrated DNA Technologies.

Reactions were run in 25 µL volumes using 1X ThermoPol PCR buffer (New England Biolabs, Ipswich, MA), 0.2 mM each dTTP, dCTP, dATP, and dGTP (Invitrogen Corporation,

Carlsbad, CA), 2 mM MgCl<sub>2</sub>, 0.1 mM each probe, 0.2 mM each primer, and 1.5 U of *Taq* polymerase (NEB). Two µL of the processed sample was used as the template in each reaction. Reactions were run in a Stratagene Mx4000 Multiplex Quantitative PCR system (Stratagene, La Jolla, CA) under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 10s and 60°C for 16s, with two endpoint fluorescence readings during each amplification segment. Positive, suspension buffer, and master mix-only controls were also included in each reaction cycle. Specificity tests were conducted in duplex reactions using the bacteria listed in Table 4-1, singly, and in mixtures.

#### *Optimization of Real-time PCR for Quantitative Analysis*

Singleplex and duplex standard curves were constructed to allow quantification of each target organism. Fresh, overnight plate cultures of *E. amylovora* Ea6-4 and *E. pyrifoliae* Ep1/96 were aseptically scraped from the plate surface and suspended in 0.01 M PB, pH 6.8 to a concentration of at least  $1 \times 10^9$  CFU/mL ( $OD_{600} = 0.6$ ) using a Beckman DU 640 spectrophotometer. The concentration of each suspension was calibrated by plating 100 µL of serial dilutions in PB and counting single colonies after 2 d. Templates for real-time PCR standard curves were prepared by serially diluting cell suspensions in clean blossom wash, either individually or as a mixture of both species. Standard curve construction was repeated using new bacterial cultures. PCR reactions were run in triplicate.

Table 4-1. Bacterial strains used in this study

Isolate	Host	Origin	Source or Reference
<i>E. amylovora</i>			
Ea6-4	<i>Pyrus</i> sp.	Canada	Gill et al., 2003
Ea1/79	<i>Cotoneaster</i> sp.	Germany	Falkenstein et al., 1988
Ea321	<i>Crataegus oxyacantha</i>	France	CFBP1367 <sup>a</sup>
IH3-1	<i>Raphiolepis indica</i>	USA	Holcomb, 1998
IL5	<i>Rubus</i> sp.	USA	McManus & Jones, 1995b
CA11	<i>Malus</i> sp.	USA	Chiou & Jones, 1991
UTRJ2*	<i>Malus</i> sp.	USA	Thomson & Ockey, 2001
Leb B66*	<i>Malus</i> sp.	Lebanon	Saad et al., 2000
NA1614-2a*	<i>Crataegus</i> sp.	Spain	Llop et al., 2006
<i>E. pyrifoliae</i>			
Ep1/96	<i>Pyrus pyrifolia</i>	Korea	Kim et al., 1999
Ep2/97	<i>Pyrus pyrifolia</i>	Korea	Jock et al., 2003b
Japanese <i>Erwinia</i> strains			
Ejp556	<i>Pyrus pyrifolia</i>	Japan	Kim et al., 2001a
Ejp557	<i>Pyrus pyrifolia</i>	Japan	Kim et al., 2001a
Ejp617	<i>Pyrus pyrifolia</i>	Japan	Kim et al., 2001a
<i>P. agglomerans</i>			
Eh21-5	<i>Pyrus</i> sp.	Canada	A. M. Svircev <sup>c</sup>
C9-1	<i>Malus</i> sp.	USA	Ishimaru et al., 1988 <sup>b</sup>
E325	<i>Malus</i> sp.	USA	Pusey, 1997 <sup>b</sup>
<i>E. carotovora</i> Ecc26			D. Cupples <sup>d</sup>
<i>E. coli</i> DH-5 $\alpha$			A. J. Castle <sup>c</sup>
<i>P. fluorescens</i> A506			Lindemann and Suslow, 1987 <sup>b</sup>
<i>P. syringae</i> pv. <i>papulans</i> 4404			A. M. Svircev <sup>c</sup>

\* strains marked with an asterisk do not contain the pEA29 plasmid

<sup>a</sup> Collection Française de Bactéries Phytopathogènes

<sup>b</sup> *P. agglomerans* C9-1 and *P. fluorescens* A506 are the active ingredients in commercial bacterial antagonists produced under the BlightBan® label by Plant Health Technologies, for the control of fire blight. *P. agglomerans* E325 is the active ingredient in a commercial bacterial antagonist produced by Northwest Agricultural Products for similar use.

<sup>c</sup> Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, Vineland Station, Canada

<sup>d</sup> Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, London, Canada

<sup>e</sup> Department of Biological Sciences, Brock University, St. Catharines, Canada

Table 4-2. Sequences, product sizes, and targets of the forward primer (F), the reverse primer (R), and the TaqMan® probe (P) used for duplex detection and quantification of *E. amylovora* (Ea) and *E. pyrifoliae* (Ep).

Oligonucleotide	Sequence (5' to 3')	Position <sup>a</sup>	Product Size
Ea-lscF	CGCTAACAGCAGATCGCA	345-362	105 bp
Ea-lscR	AAATACGCGCACGACCAT	449-432	
Ea-lscP	(CY5)CTGATAATCCGCAATTCCAGGATG(IAbRQ) <sup>b</sup>	366-389	
Ep-hrpwF	CGCTAACCCGACTGTGCT	756-773	77 bp
Ep-hrpwR	TGAAGGTTTGCCCTTTGC	832-815	
Ep-hrpwP	(FAM)ATGACACCATCATCGTAAAGGCGG(BHQ-1) <sup>b</sup>	776-799	

<sup>a</sup> Binding positions are based on GenBank files X75079 (*lsc*) and AY237642 (*hrpW*).

<sup>b</sup> IAbRQ and BHQ-1 are the manufacturer's proprietary quencher molecules.



### *E. amylovora* and *E. pyrifoliae* Competition Assays

Bartlett pear shoots bearing dormant buds were harvested in late winter. Shoots were forced to bloom by placing in water at 23°C, in a well-lit room. Newly opened blossoms with yellow, undehisced anthers were harvested by hand and individually placed into scintillation vials such that the peduncle extended through a hole drilled in the lid and into the sterile tap water contained in the vial.

Suspensions of *E. amylovora* and *E. pyrifoliae* were prepared by aseptically scraping cells from fresh, overnight cultures into 0.01 M PB, pH 6.8. Suspensions were adjusted to  $1 \times 10^9$  CFU/mL ( $OD_{600} = 0.6$ ) using a Beckman DU 640 spectrophotometer, stored on ice, and further diluted in PB as needed immediately prior to application.

Ten  $\mu$ L of a bacterial suspension was inoculated directly onto the hypanthium. Treatments were: 0.01 M PB, pH 6.8 (negative control);  $1 \times 10^6$  CFU/blossom of either *E. amylovora* or *E. pyrifoliae* (positive control for virulence);  $1 \times 10^4$  CFU/blossom of *E. amylovora*, or *E. pyrifoliae*, or a 1:1 mixture of both for a total bacterial count of  $1 \times 10^4$  CFU/blossom;  $1 \times 10^2$  CFU/blossom of *E. amylovora*, or *E. pyrifoliae*, or a 1:1 mixture of both for a total bacterial count of  $1 \times 10^2$  CFU/blossom. Each treatment was applied to 20 blossoms. Following inoculation the blossoms were loosely sealed in large plastic bins containing about 500 mL of water to maintain a high relative humidity and incubated at 23°C.

Ten blossoms from each treatment were assessed after 3 d, and another 10 blossoms after 5 d. Each blossom was removed from the scintillation vial using sterile forceps. Disease symptoms were scored using a severity index (0, no necrosis; 1, necrosis on the stigma and hypanthium; 2, necrosis visible on the immediate underside of the blossom; 3, necrosis extends

into the ovary, no farther than the widest point; 4, necrosis extends to the base of the ovary; 5, necrosis extends into the peduncle). Every second blossom was further processed in order to quantify surface bacterial populations (see below). The experiment was conducted three times using suspensions prepared from independent bacterial cultures.

#### *Assessment of Bacterial Numbers on Blossoms*

After symptom severity was assessed, the petals and peduncle were removed and the remaining tissue (ovary, hypathium, stamens, stigma) was placed in a sterile 1.5 mL microcentrifuge tube. One mL of Direct Plant Extraction Buffer (DiPEB, Cat. No. 00690, Agdia Inc., Elkhart, IN) was added to each tube. Tubes were mixed by vortexing briefly, placed in a water bath sonicator such that only the cap was not immersed, and sonicated for 3 min to dislodge bacterial cells. Plant tissue was removed using a sterile toothpick and discarded. Bacterial cells were concentrated 10-fold by centrifuging the blossom wash at 13 000 xg for 5 min, decanting the supernatant, and resuspending the pellet in 100  $\mu$ L of DiPEB. This processed sample was used directly as the template for real-time PCR. This procedure was also conducted using uninoculated blossoms, and the resulting clean blossom wash was used as a negative control.

#### *Statistical Analysis*

Regression analysis of standard curve data was conducted in SigmaPlot, version 8 (SPSS, Inc., Chicago, IL). All other data were analyzed using SAS (Statistical Analysis Systems 8.2; SAS Institute, Cary, NC). Disease severity index ratings from the competition assay were

converted to a percent scale based on the total hypanthium and ovary surface area showing visible necrosis. Population data were logarithmically transformed (base 10) in order to produce normally distributed data suitable for further analysis. If a given pathogen species was not detected on a blossom, the population size was adjusted from 0 to 2 CFU in order to avoid undefined log functions or ratios of logs. Disease severity data and transformed population data were analyzed with the general linear model (PROC GLM), using the Tukey-Kramer mean separation test for multiple comparisons.

To assess whether competition occurred between *E. amylovora* and *E. pyrifoliae*, their growth on independent blossoms was compared to their growth on the same blossom. Each blossom inoculated with only *E. amylovora* was paired randomly with a blossom from the same replicate that had been inoculated with only *E. pyrifoliae*. The "relative growth performance" of each species was expressed as:

$$\log(\text{species 1 alone}) / [\log(\text{species 1 alone}) + \log(\text{species 2 alone})], \text{ or}$$

$$\log(\text{species 1 in mixture}) / [\log(\text{species 1 in mixture}) + \log(\text{species 2 in mixture})].$$

For each species, relative growth performance alone was compared to relative growth performance in a mixture using a t-test with a Satterthwaite adjustment for unequal variances. There was no significant difference between replicated experiments ( $P > 0.05$ ), therefore data from multiple replications were pooled. Where data were not normally distributed ( $P < 0.05$  in a Kolmogorov-Smirnov test), the results of parametric tests and non-parametric tests were compared. In no case was the departure from normality sufficient to invalidate the parametric tests. All mean values are given as mean  $\pm$  95% confidence limits.

## Results

### *Specificity and Sensitivity of Duplex Real-time PCR*

The specificity of levansucrase (*lsc*) probe and primers was tested using all bacterial isolates and species listed in Table 4-1. All *E. amylovora* isolates were positive regardless of the presence or absence of the pEA29 plasmid. No cross reactions were observed with *E. pyrifoliae* strains or any other species. The *hrpW* probe and primers detected all *E. pyrifoliae* strains as well as the Japanese *Erwinia* strains. This combination did not detect any *E. amylovora* isolate or any other species. No change in threshold cycle ( $C_T$ ) values were observed in the presence of non-target species.

Fluorescence signals were regularly obtained from standardized samples containing as few as 20 CFU of either *E. amylovora* or *E. pyrifoliae* in a 25  $\mu$ L reaction. This detection threshold occurred at an approximate  $C_T$  value of 33. Reaction efficiency, as calculated by the Stratagene Mx4000 software, was greater than 95 %.

Duplex standard curves for *E. amylovora* and *E. pyrifoliae* are shown in Figure 4-1. Concentrations higher than  $5 \times 10^6$  CFU per 25  $\mu$ L reaction were not tested. For *E. amylovora*, the relationship between ( $C_T$ ) and initial quantity was linear across a 4.5-log range. For *E. pyrifoliae*, the relationship between  $C_T$  and initial quantity was linear across a 5-log range. While detection, indicated by a fluorescence signal by 33 cycles, was reliable and for initial pathogen quantities above 20 CFU per 25  $\mu$ L reaction, quantification of both pathogens was highly repeatable only for initial quantities greater than approximately 100 CFU per reaction. The standard curves for both species are essentially identical; the slight differences between slope and intercept values were within the range of variation between replicate real-time PCR runs.

Standard curves were prepared from cells diluted in buffer that had been sonicated with uninoculated blossoms according to the sampling procedure used for the competition assays. The curves therefore correspond directly to initial cell numbers, and account for the presence of any PCR inhibitors that may be present in the experimental samples. Since blossom infection occurs through the stigma and hypanthium, petals and peduncles were removed from the blossom, and bacteria were collected from the surfaces of the remaining structures. Removal of petals and peduncles improved the sensitivity of bacterial quantification (data not shown), presumably because it reduced the total volume of plant tissue, and thus the concentration of plant-derived PCR inhibitors. DNA extraction was not performed as part of sample preparation. The components of the wash buffer, along with the 5 min initial denaturation cycle at 95°C, were sufficient to make the bacterial target DNA accessible for amplification.

There is no substantial difference between the standard curves produced by singleplex and duplex reactions (data not shown). For a given target species, duplex detection of a given cell concentration occurred about 0.5  $C_T$  later than if the same sample was tested in a singleplex reaction. However this difference is within the range of variation between replicate runs. Sensitivity did not differ between duplex and singleplex reactions.

#### *Virulence of E. amylovora and E. pyrifoliae*

Disease severity in blossoms treated with either *E. amylovora* or *E. pyrifoliae* alone was modelled in terms of pathogen identity, initial inoculum size, and time elapsed since inoculation. Across the experiment as a whole, initial inoculum size and time elapsed since inoculation were significant predictors of disease severity ( $P < 0.0001$  for both). There was also a significant

interaction between these two factors ( $P = 0.0075$ ). Specifically, initial inoculum size showed a significant ( $P < 0.0001$ ), but qualitatively different, effect on disease severity at each of the two sampling times. Table 4-3 shows the results of a virulence assessment of *E. amylovora* and *E. pyrifoliae* on Bartlett pear blossoms. Blossoms showed no disease symptoms at the time of inoculation. In general, mean disease severity increased with increasing inoculum. Three days after inoculation, blossoms inoculated with  $1 \times 10^6$  CFU of either pathogen showed significantly more severe symptoms than blossoms that had received a smaller inoculum ( $P < 0.01$ ). The differences between blossoms treated with  $1 \times 10^4$  CFU,  $1 \times 10^2$  CFU, and 0 CFU are less clear, as the difference between the 0 CFU and  $1 \times 10^4$  CFU treatments is just significant at the 5% level ( $P = 0.0496$ ). By day 5, however, the only significant difference in disease severity was between inoculated and uninoculated blossoms ( $P < 0.0001$ ).

There was no significant effect of pathogen identity on disease severity across the experiment as a whole ( $P = 0.5182$ ), or within any level of initial inoculum size at either sampling time (all  $P > 0.4100$ ).

#### *Effect of Initial Pathogen Ratio on Disease Severity*

Figure 4-2 shows the overall effect of initial pathogen ratio on disease development, both 3 and 5 d after inoculation. Regardless of the time of observation, within each inoculum level, there is no significant difference between the severity of symptoms in blossoms treated with a 1:1 mixture of both pathogens and the severity of symptoms in blossoms treated with the same total amount of either species alone.

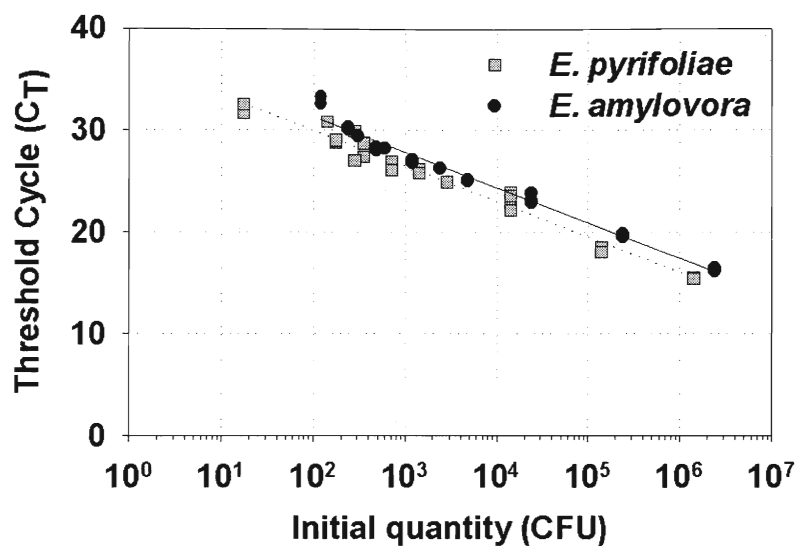


Figure 4-1. Duplex standard curves for the quantitative detection of *E. amylovora* and *E. pyrifoliae*. Known amounts of each pathogen were diluted in clean blossom wash. Quantification of *E. amylovora* (solid line) is given by  $y = 38.4 - 3.48 \log(x)$ ,  $R^2 = 0.975$ . Quantification of *E. pyrifoliae* (dotted line) is given by  $y = 37.0 - 3.48 \log(x)$ ,  $R^2 = 0.973$ .

### *Relative Population Sizes of E. amylovora and E. pyrifoliae*

Bartlett pear blossoms were inoculated with *E. amylovora*, *E. pyrifoliae*, or a 1:1 mixture of the two. Real-time PCR was used to determine the population sizes of both species after 3 d and again after 5 d. The logarithmically transformed population sizes were modelled in terms of pathogen identity, whether the pathogen was inoculated alone or as a mixture, initial inoculum size, and time elapsed since inoculation. There was no difference in population sizes between days 3 and 5 ( $P = 0.5491$ ), or between different levels of initial inoculum ( $P = 0.3414$ ). Pathogen identity was significant ( $P < 0.0001$ ), as was mixture ( $P = 0.0003$ ), and there was a significant interaction between these two factors ( $P = 0.0021$ ).

Figure 4-3A shows the mean population sizes of *E. amylovora* and *E. pyrifoliae* on the pear blossoms, when present alone or together. In blossoms inoculated with only one of the two pathogen species, *E. amylovora* populations grew to a greater size than did *E. pyrifoliae* populations ( $P = 0.0002$ ). *E. amylovora* populations were not significantly smaller on blossoms inoculated with both species than they were when *E. pyrifoliae* was absent ( $P = 0.6688$ ). However, *E. pyrifoliae* population sizes were significantly smaller in the presence of *E. amylovora* than when they grew alone on the blossoms. This difference is more clearly demonstrated by the relative growth performance index (Figure 4-3B), where the growth of a species in either situation was expressed in relation to the total growth of both species. The relative contribution of *E. amylovora* to total bacterial growth was significantly greater ( $P = 0.0001$ ), and the relative contribution of *E. pyrifoliae* was significantly less ( $P = 0.0001$ ), on blossoms inoculated with both species than on blossoms inoculated with a single species.



Table 4-3. The effect of initial inoculum size on disease severity in pear blossoms.

Time Frame	Initial Inoculum Size (CFU/blossom)	Percent Disease <sup>a</sup> (mean ± SD)
Across Entire Experiment	0	12.5 <sup>A</sup>
	1 x 10 <sup>2</sup>	38.8 <sup>B</sup>
	1 x 10 <sup>4</sup>	48.2 <sup>B</sup>
	1 x 10 <sup>6</sup>	64.4 <sup>C</sup>
3 days Elapsed Since Inoculation	0	5.0 <sup>A</sup>
	1 x 10 <sup>2</sup>	13.4 <sup>A,B</sup>
	1 x 10 <sup>4</sup>	23.3 <sup>B</sup>
	1 x 10 <sup>6</sup>	46.0 <sup>C</sup>
5 days Elapsed Since Inoculation	0	20.0 <sup>A</sup>
	1 x 10 <sup>2</sup>	64.2 <sup>B</sup>
	1 x 10 <sup>4</sup>	73.1 <sup>B</sup>
	1 x 10 <sup>6</sup>	82.8 <sup>B</sup>

<sup>a</sup> Disease severity is expressed as percent of surface area that is visibly necrotic. Values are the means of two experiments, each with 10 blossoms per treatment. Within each time frame, means with the same letter are not significantly different ( $P < 0.05$ , Tukey-Kramer test for multiple comparisons).

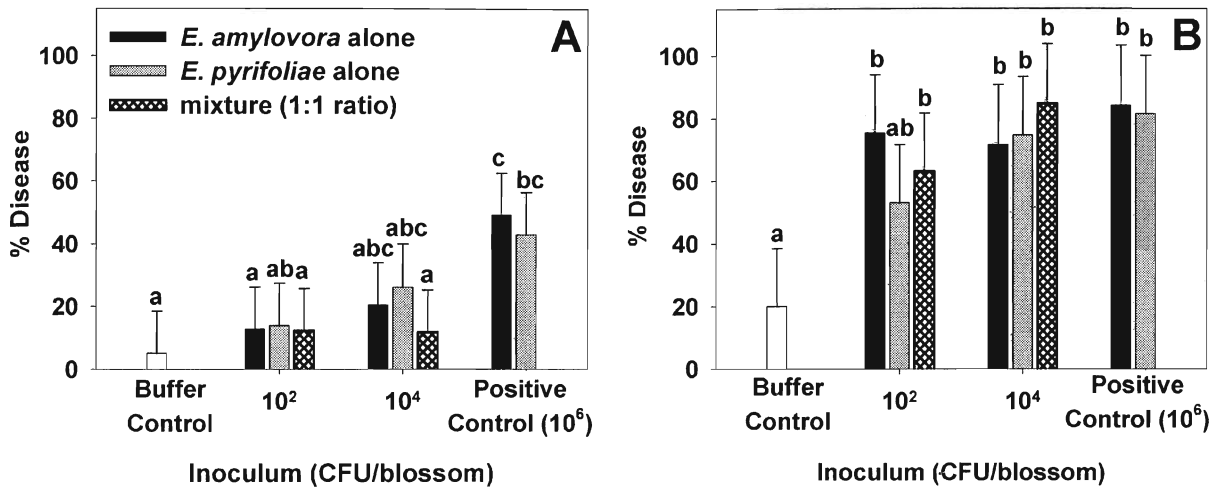


Figure 4-2. Effect of initial pathogen ratio on disease severity in blossoms inoculated with *E. amylovora* only, *E. pyrifoliae* only, or a 1:1 mixture of both. Percent Disease is shown as mean + 95% confidence limit for A: 3 d after inoculation, B: 5 d after inoculation. On each day, means with the same letter are not significantly different ( $P < 0.05$ ).

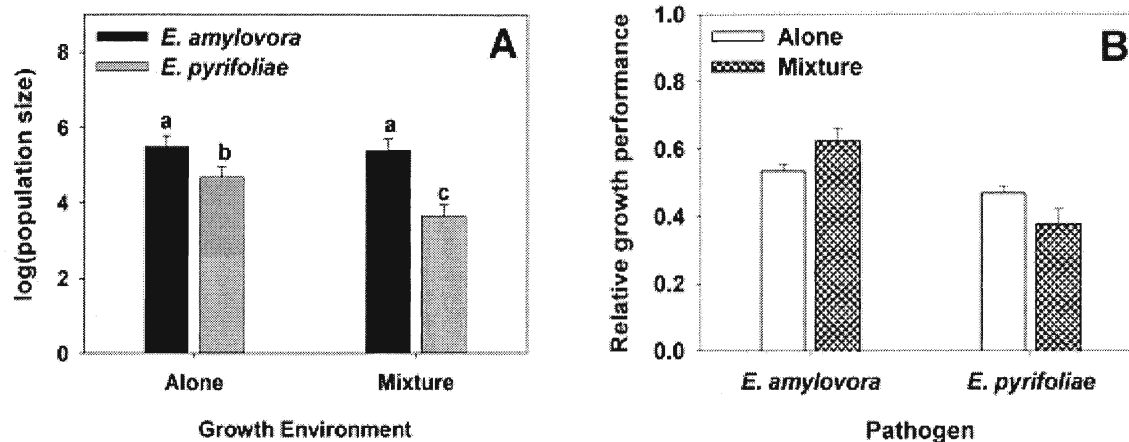


Figure 4-3. A: Population sizes of *E. amylovora* and *E. pyrifoliae* on pear blossoms inoculated with each species alone, or with a 1:1 mixture of both species. Populations are given as mean + 95% confidence limits. Means with the same letter are not significantly different ( $P < 0.01$ ). B: Relative growth performance of *E. amylovora* and *E. pyrifoliae* when growing on Bartlett pear blossoms, alone or in a mixture. Values are mean + 95% confidence limits.

## Discussion

Fire blight causes economically significant losses of rosaceous fruit crops, particularly apple and pear, in many parts of the world. The causative agent, *E. amylovora*, is the focus of intensive research aimed at understanding and inhibiting its epiphytic and endophytic growth phases. *E. pyrifoliae* is very closely related to *E. amylovora*, and causes a similar disease on Asian pear trees. A rapid, sensitive, molecular method of detecting and quantifying these phytopathogens will allow more convenient detection, and more accurate monitoring of their *in vivo* growth characteristics. In this study, a duplex real-time PCR method was developed that allows simultaneous identification and quantification of *E. amylovora* and *E. pyrifoliae* directly from blossom surfaces, independent of their cultivability.

Previous PCR methods for *E. amylovora* detection have been based on the pEA29 plasmid (McManus, Jones, & Bonn, 1996; Llop et al, 2000; Salm & Geider, 2004). A chromosomal target was chosen for this study because of the emergence of *E. amylovora* isolates in nature that lack pEA29 (Llop et al., 2006). Specifically, the levansucrase gene (*lsc*) was selected as the target region since levan is commonly produced by *E. amylovora* strains as a second exopolysaccharide and plays significant role in virulence (Gross, Geier, Rudolph, & Geider, 1992; Geier & Geider, 1993). *E. pyrifoliae* and Japanese *Erwinia* strains fail to produce levan on sucrose-amended media (Kim et al., 2001a). Therefore, in order to differentiate *E. amylovora* from *E. pyrifoliae*, *lsc* was selected as an amplification target.

Specific quantification of *E. pyrifoliae* was achieved with primers and a probe that amplify and detect a 77 bp region of the *hrpW* gene. This gene is part of the *hrp* cluster, which is present on the chromosome of both *E. amylovora* and *E. pyrifoliae*, but which shows sufficient

interspecies variation among other *hrp* genes to permit reliable differentiation of *E. pyrifoliae*. The detection of the Japanese *Erwinia* isolates with a system based on differences between *E. amylovora* and *E. pyrifoliae* is further evidence that these isolates should not be considered variants of *E. amylovora*, but may well belong to *E. pyrifoliae*. This is supported by data from other studies, which indicate that these isolates are not identical to *E. amylovora*, and, in fact, resemble *E. pyrifoliae* more closely than *E. amylovora* (Kim et al., 2001a; Jock et al., 2003a; Matsuura et al., 2007).

Fluorescence signals were regularly obtained from standards containing as few as 20 CFU per 25  $\mu$ L reaction ( $C_T = 33$ ). Quantification was very reliable for cell concentrations as low as 100 CFU per 25  $\mu$ L reaction ( $C_T = 30$ ). For the purposes of qualitative detection, samples yielding a  $C_T$  value of 30 or less would be considered positive for the respective pathogen, while samples yielding a  $C_T$  value between 30 and 35 should be considered suspect and re-tested. During the population monitoring experiments described in this study,  $C_T$  values less than 26 were very rarely obtained. Despite the fact that the primers and probes described here target single-copy chromosomal genes, the sensitivity of the method developed in this study is comparable to that achieved in a pEA29-based real-time PCR assay for *E. amylovora* alone (Salm & Geider, 2004).

One of the major advantages of the primers and probes developed in this study is that they are compatible in a duplex reaction, allowing simultaneous, independent quantification of both *E. amylovora* and *E. pyrifoliae*. The primers and probes developed in this study were used to investigate the population dynamics of *E. pyrifoliae* on the blossom surface, and to study the interactions of *E. pyrifoliae* and *E. amylovora* on a common host plant. The blossom bioassay

used for these experiments was based on the fact that primary infection by *E. amylovora* occurs through the reproductive organs (van der Zwet & Keil, 1979). Therefore each treatment group consisted of a series of isolated blossoms that were individually inoculated and assessed.

In blossoms treated with a single pathogen species, there was no apparent difference between the virulence of *E. amylovora* and *E. pyrifoliae* on Bartlett pear blossoms. No more than 100 cells of either pathogen were needed to cause fire blight symptoms in a single blossom, and there was no difference in the progression of symptoms resulting from inoculation with a given amount of either pathogen. However, the mean population size of *E. pyrifoliae* on the blossom surfaces was significantly smaller than the mean *E. amylovora* population size, which suggests that *E. pyrifoliae* does not grow as well as *E. amylovora* on Bartlett pear blossoms. It may be that this difference was not sufficient to hinder the infection process. This suggestion of a threshold population size for infection is consistent with a report from Johnson et al (1993b) that incidence of fire blight in Bartlett pear orchards was associated with the number of blossoms that supported *E. amylovora* populations greater than  $1 \times 10^5$  CFU/blossom.

In blossoms treated with a 1:1 mixture of both *E. amylovora* and *E. pyrifoliae*, disease severity was not significantly different than in blossoms inoculated with either species alone. Clearly the presence of both species together neither amplifies nor inhibits their pathogenicity. What is not clear from these results is whether the two species are contributing equally to the production of disease, or whether one species outcompetes the other to become dominant. Real-time PCR data revealed that the growth of *E. amylovora* on Bartlett pear blossoms was unaffected by the presence of an equal number of *E. pyrifoliae* cells. In contrast, the growth of *E. pyrifoliae* was negatively affected by *E. amylovora* and the population size of *E. pyrifoliae*

relative to *E. amylovora* was significantly smaller when both species were present together than when they grew alone. This observation suggests that *E. amylovora* has greater competitive fitness on Bartlett pear blossoms. It is not known whether this simply represents differential success in the utilization of available nutrients and other resources, or whether any specific antagonism is involved.

It would be interesting to test whether the relative fitness of these pathogens is the same on blossoms of *Pyrus pyrifolia*, or whether *E. pyrifoliae* is better adapted to Asian pears than *E. amylovora*. *E. amylovora* and *E. pyrifoliae* are not currently known to co-exist in nature, but they are very closely related and have partially overlapping host ranges. It is generally accepted that *E. amylovora* is native to North America (Bonn & van der Zwet, 2000), but nothing is known about the evolutionary relationship between *E. amylovora* and *E. pyrifoliae* beyond the substantial genomic similarity. An understanding of how they behave when they are present together on different host species could shed light on their current distribution or potential distribution in the future. In light of the rapid spread of modern plant pathogens, it is certainly possible that these species could be introduced to the same regions in the future. Their relative fitness will influence the outcome of such introductions.

The methods developed in this study are transferable to a field situation, and have been successfully used to monitor *E. amylovora* populations in experimental orchards as part of a fire blight biological control study (Kim, Lehman, Castle, & Svircev, unpublished data). The omission of a DNA extraction step makes this technique attractive in terms of time- and cost-savings, especially for field studies, which tend to rapidly generate a large number of samples.

The potential for simultaneous quantification of several target species is not limited to *E.*

*amylovora* and *E. pyrifoliae*. The methods described here should be easily applicable to simultaneous monitoring of a pathogen and biocontrol agent. The primers and probes developed in this study are species-specific. Strain-specific real-time PCR has been used to monitor the population of an applied fire blight biocontrol agent, *Pseudomonas fluorescens* EPS62e (Pujol, Badosa, Manceau, & Montesinos, 2006). The combination of species-specific and strain-specific primers and probes could be used to study the relative contribution of an applied biocontrol agent to the total population of that species. Ultimately, since all primers and probes must function at the same reaction temperature, the applicability of multiplex real-time PCR to phytopathology, in general, and biocontrol, in particular, will depend on the availability of a large amount of sequence data.

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### **Part III: Development and Evaluation of the Phage-Carrier Biopesticide**

## Chapter 5: Evaluation of the interactions between *Erwinia amylovora*, *Pantoea agglomerans* Eh21-5, and *Erwinia* phages

### Abstract

The *in vitro* and *in planta* growth characteristics and interactions of *E. amylovora*, *P. agglomerans*, and *E. amylovora* phages were studied in order to establish parameters for treatment preparation and application during field trials. *In vitro* growth curves were constructed for *P. agglomerans* Eh21-5, *E. amylovora* Ea6-4 and Ea29-7, and two *Erwinia* phages. With adequate aeration, the doubling time of exponentially growing *E. amylovora* and *P. agglomerans* is approximately 45 min in nutrient broth at 27°C. The latency period of  $\phi$ Ea31-3 in Ea110 is approximately 1.5 h, but the initial rate of adsorption is slow. The *in planta* interactions among *E. amylovora*, the *P. agglomerans* carrier, and four *Erwinia* phages were also studied. Four phages were used to test the effects of treatment timing and multiplicity of infection on biocontrol efficacy using the pear blossom assay. Phage-carrier combinations were more effective at reducing fire blight symptom severity than the carrier alone if 3 h (vs. 0 h) was allowed to elapse between treatment application and pathogen application.

## Introduction

The replication of phages is very dependent upon the metabolic condition of the host cell, and by extension the environment in which the cell exists. The environment on a pear or apple hypanthium is very different from *in vitro* conditions, but both are relevant to the development of a phage-based biopesticide. The biopesticide components must be effective in the field, but they must also be prepared in a laboratory, be it on a research or an industrial scale.

Optimizing the propagation of phages in liquid culture requires an understanding of a phage's growth characteristics. These are different for each phage-host combination and set of incubation conditions, but even a general idea of the growth cycle can be helpful. Adsorption of tailed phages to Gram-negative bacteria is frequently based on components of the outer membrane, and may require the presence of certain cofactors in the surrounding environment (Kutter, Raya, & Carlson, 2005).

Adsorption can be a multi-stage process in which reversible interactions involving host recognition and virion positioning are followed by irreversible binding of phage proteins to receptors on the host cell, penetration of the cell wall and cell membrane, and transfer of the phage genome into the host cell (Kutter, Raya, & Carlson, 2005).

Irreversible binding marks the beginning of the “eclipse” period, during which artificial lysis of the host cell will not result in the release of infective phages, since the infecting phage can not free itself to infect another cell, and no new virions have been produced. The eclipse period lasts until the first progeny phages have been assembled within the cell. After this point, artificial lysis of the cell will cause the release of an increasing number of intact and infective progeny virions. The time between irreversible phage adsorption and phage-directed lysis of the

host is called the latent period. The average number of progeny phages released by a single infected cell due to phage-directed lysis is known as the burst size, and can vary substantially among specific phage-host combinations.

Since phage DNA replication and gene expression depend on host components such as ribosomes and raw materials, the length of the eclipse and latency periods can be affected by the nutritional status and metabolic activity of the cell (Kutter, Raya, & Carlson, 2005). The efficient propagation of phages therefore requires an understanding of the optimum growth conditions of both phages and their host bacteria.

Once propagated, phages that are to be used therapeutically must then be stored in a manner that is consistent with the conditions of their eventual use, and that maintains a stable population of viable virions.

To address the issues of propagation and preservation, the *in vitro* growth and survival characteristics of some of the bacteria and phages to be used in field trials were examined. Growth curves were constructed for the carrier, *P. agglomerans*, and for two of the strains of *E. amylovora* on which phages will be propagated. The length of the latent period in phage-infected *E. amylovora* liquid cultures was studied. In addition, the long-term survival of morphologically different phages was monitored in liquid media in which high-titre phage stocks might be stored, and in ionic solutions that may be used to encapsulate phages as part of a field delivery system. To facilitate the transfer of the phage-carrier biopesticide into the field, the pear blossom bioassay was used to evaluate the efficacy of phage-carrier combinations in reducing the severity of *E. amylovora*-induced necrosis, and to evaluate how MOI and prior establishment of microbe populations influence that efficacy.

## Methods

### *Bacteria, Phages, and Growth Media*

Media and growth conditions were as described in Chapter 2, unless otherwise indicated.

Table 5-1 lists the bacteria and phages used in this experiment. Isolate origins are described in Table 2-1 and Table 2-2.

### *Bacterial Growth Curves*

The *in vitro* growth of *E. amylovora* Ea6-4, *E. amylovora* Ea29-7, and *P. agglomerans* Eh21-5 was studied by monitoring their populations in pure liquid culture. One litre flasks containing 500 mL of nutrient broth were inoculated with 10 mL of a bacterial suspension prepared to  $1 \times 10^9$  CFU/mL in PB. The cell concentration was monitored by spectrophotometry, specifically, the absorbance of light at 600 nm. Growth curves were repeated at least twice, using independent bacterial cultures.

Two different experiments were conducted. In the first, *E. amylovora* Ea6-4 and *P. agglomerans* Eh21-5 were studied, and the flasks were incubated on an orbital shaker at 150 rpm. In the second *E. amylovora* Ea6-4 and *E. amylovora* Ea29-7 were studied, and the flasks were incubated in a water bath. In this second experiment, there was very little constant motion, but the flasks were vigorously shaken when samples were taken every hour. Within each experiment, at least 3 replicate flasks per species were grown, each on different days.

Spectrophotometric readings of cell concentration were calibrated by simultaneously plating serial dilutions of Ea6-4 and Eh21-5 on nutrient agar.

Table 5-1. Bacteria and phages used in this study.

Strain	Bacterial Host	Used For
<i>Erwinia amylovora</i>		
Ea6-4	NA	Bacterial growth curves; Blossom assays
Ea29-7	NA	Bacterial growth curves
Ea110	NA	Phage growth curves
<i>Pantoea agglomerans</i>		
Eh21-5	NA	Bacterial growth curves; Blossom assays
Phages		
φEa9-5	Ea-4	Blossom assays
φEa21-4	Ea6-4	Blossom assays
φEa31-3	Ea29-7	Phage growth curves
φEa35-4	Ea110	Phage growth curves
φEa35-5	EaD-7	Blossom assays
φEa46-1A2	EaD-7	Blossom assays

### *Phage Growth Curves*

Phage growth curves were constructed using a modification of the method described by Carlson (2005). Ten millilitres of a  $1 \times 10^9$  CFU/mL suspension of the bacterial host was added to each of two 1 L flasks containing 480 mL of nutrient broth. The flasks were incubated in a water bath at 27°C, and shaken vigorously at least every 30 min to ensure adequate aeration. When the bacterial culture reached a concentration of approximately  $1 \times 10^8$  CFU/mL,  $\phi$ Ea31-3 was added to one flask for a final concentration of  $1 \times 10^9$  PFU/mL, and an equivalent volume of nutrient broth was added to the uninfected control culture. Immediately, and every 30 min thereafter, each flask was shaken well, and a 100  $\mu$ L sample was taken. These samples were serially diluted in nutrient broth. The concentration of bacterial survivors at each time point was determined by spreading a 100  $\mu$ L aliquot from the  $10^{-5}$ ,  $10^{-6}$ , and  $10^{-7}$  dilutions over a nutrient agar plate, and counting the number of colonies that formed after 2 d. The total concentration of infective centres was determined by mixing a 100  $\mu$ L aliquot from the same dilutions with 100  $\mu$ L of  $1 \times 10^9$  CFU/mL Ea110 and 3 mL of molten top agar to form an agar overlay. The number of plaques was counted after 1 d of growth.

### *Phage Survival in Liquid Media*

The long-term survival of  $\phi$ Ea46-1A2 in liquid media stored at 4°C was tested. Six media were used: nutrient broth, 10 mM PB (pH 6.8) amended with 100 mM NaCl and 2 mM  $\text{MgCl}_2$ , reverse osmosis water, autoclaved reverse osmosis water, municipal tap water, autoclaved municipal tap water. Filtered  $\phi$ Ea46-1A2 lysates were mixed with the desired suspension medium in a 3:1 ratio and subjected to normal flow diafiltration at 4°C using the Amicon

apparatus described in Chapter 7. The volume of each suspension was reduced from 2 L to 300 mL, an additional 600 mL of the diluent was added to the stirred cell, and the retentate was reduced to a final volume of 300 mL. The result was a 12-fold dilution of dissolved substances, with a 30% increase in phage concentration. The concentration of viable phage in each medium was estimated by serially diluting an aliquot of each in nutrient broth, and plating 100  $\mu$ L of each dilution according to the soft agar overlay technique described in Chapter 2. All of the suspensions still had a yellow tinge following diafiltration, and so they were diluted 10-fold in their respective diluents immediately before being plated at the 13-week point.

The survival of  $\phi$ Ea31-3 and  $\phi$ Ea46-1A2 was also tested in 0.015 M, 0.15 M, and 0.5 M NaCl. A  $3.0 \times 10^9$  PFU/mL suspension of  $\phi$ Ea46-1A2 in nutrient broth was diluted 100-fold in each saline solution. The same was done with a  $2.1 \times 10^9$  PFU/mL suspension of  $\phi$ Ea31-3. The saline suspensions were stored at 4°C. The concentration of viable phage in each medium was determined after 24 h and after 11 weeks.

#### *Optimization of Phage-Carrier Application to Blossoms*

The efficacy of phage-carrier mixtures was tested using the blossom assay described in Chapter 2. Treatments were pipetted directly on to the hypanthium in 10  $\mu$ L volumes. Treatments consisted of the buffer control, the pathogen-free control, carrier alone, or carrier-phage combinations. Each carrier-phage combination was prepared at two different multiplicities of infection, 0.5 or 0.05, with  $5 \times 10^7$  CFU/mL of carrier. Three hours after treatment application, the blossoms were challenged by applying 10  $\mu$ L of a  $1 \times 10^8$  CFU/mL suspension of *E. amylovora* Ea6-4. The entire experiment was repeated with no elapsed time between treatment



application and pathogen application. Blossoms were incubated at room temperature with high relative humidity. Disease symptoms were evaluated after 4 d.

### *Statistical Analysis*

Regression analysis of exponential bacterial growth was conducted in Excel 2003 (Microsoft Corporation, Redmond, WA). All other data were analyzed using SAS (Statistical Analysis Systems 8.2; SAS Institute, Cary, NC). The stability of viable phages in each medium was analyzed by regression of the logarithmically transformed concentration data on elapsed time (PROC GLM). The distribution of residuals showed that the assumption of homoscedasticity was not violated. Disease severity data from the blossom assays were converted to a percent scale based on the total hypanthium and ovary surface area showing visible necrosis. The severity data were then analyzed with the general linear model (PROC GLM) using the Tukey mean separation test for multiple comparisons.

## Results

### *Bacterial Growth Curves*

*E. amylovora* and *P. agglomerans* spectrophotometric calibration curves are given in Figure 5-1. There is a slight difference between the two curves, but in both cases an OD<sub>600</sub> of 0.6 corresponds to  $1 \times 10^9$  CFU/mL.

Figure 5-2 shows the growth of *E. amylovora* Ea6-4 and *P. agglomerans* Eh21-5. For both species, the exponential growth phase commenced approximately 3 h after the media was inoculated, and lasted for approximately 4 h. During exponential growth, the number of cells present at time  $t$  is defined as  $N_t = N_0 e^{kt}$ , where doubling time is equal to  $\ln(2)/k$ . Based on regression analysis of the exponential growth phase, the doubling times of *E. amylovora* Ea6-4 and *P. agglomerans* Eh21-5 were 44 and 48 minutes, respectively. Similar results were obtained in the second experiment, with exponential growth beginning approximately 3 h after inoculation, and ceasing 7 or 8 h after inoculation. However, the doubling times of *E. amylovora* Ea6-4 and *E. amylovora* Ea29-7 were longer, 130 min and 109 min, respectively (data not shown).

### *Phage Growth Curves*

The results of the phage growth experiments are shown in Figure 5-3. Very similar results were obtained in both trials. Cell concentration in the control cultures continued to increase throughout the course of the experiment, with an initial doubling time of about 90 min. Cell growth reached a plateau after about 3 h, or 9 h after the culture was first inoculated. By comparison, cell concentrations in the infected cultures were about 5-fold lower than in the

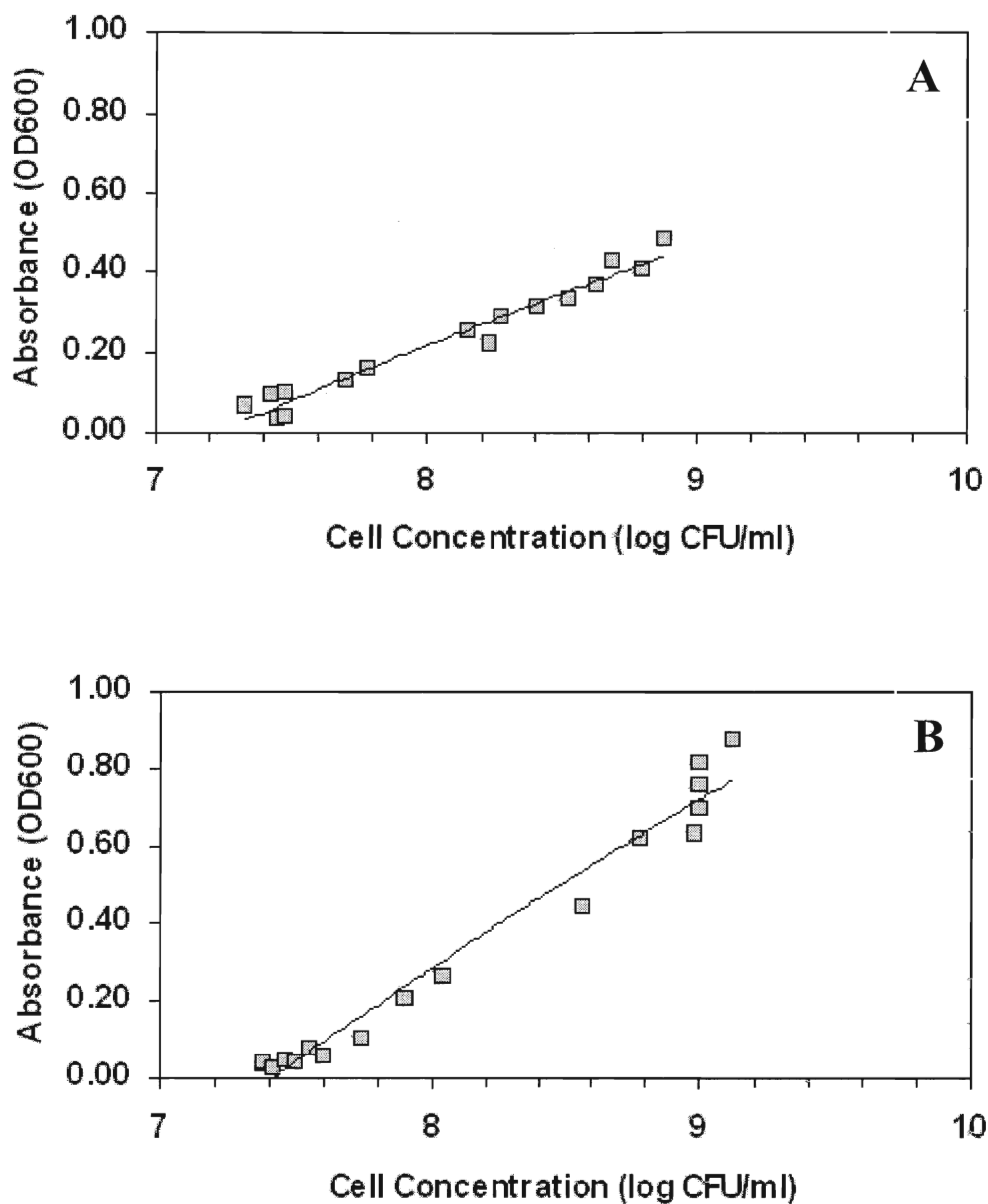


Figure 5-1. Calibration curves for spectrophotometric determination of A) *E. amylovora* Ea6-4, and B) *P. agglomerans* Eh21-5 cell concentration in liquid culture. Regression of absorbance on cell concentration is described by  $y = 0.115 \ln(x) - 1.90$ ,  $R^2 = 0.967$  for *E. amylovora*, and  $y = 0.197 \ln(x) - 3.36$ ,  $R^2 = 0.974$  for *P. agglomerans*.

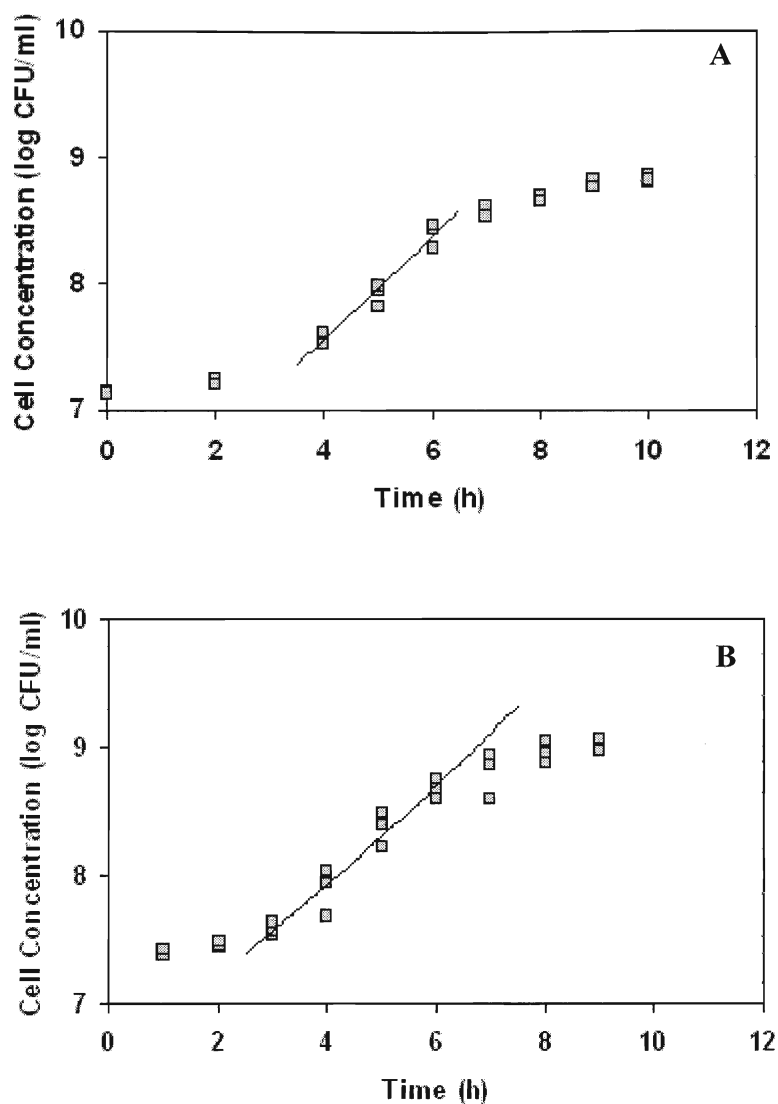


Figure 5-2. Growth of A) *E. amylovora* Ea6-4 and B) *P. agglomerans* Eh21-5 in liquid culture at 27°C. The exponential growth phase is described by  $y = (8.4 \times 10^5)e^{0.938x}$ ,  $R^2 = 0.951$  for *E. amylovora* and  $y = (3 \times 10^6)e^{0.864x}$ ,  $R^2 = 0.936$  for *P. agglomerans*.

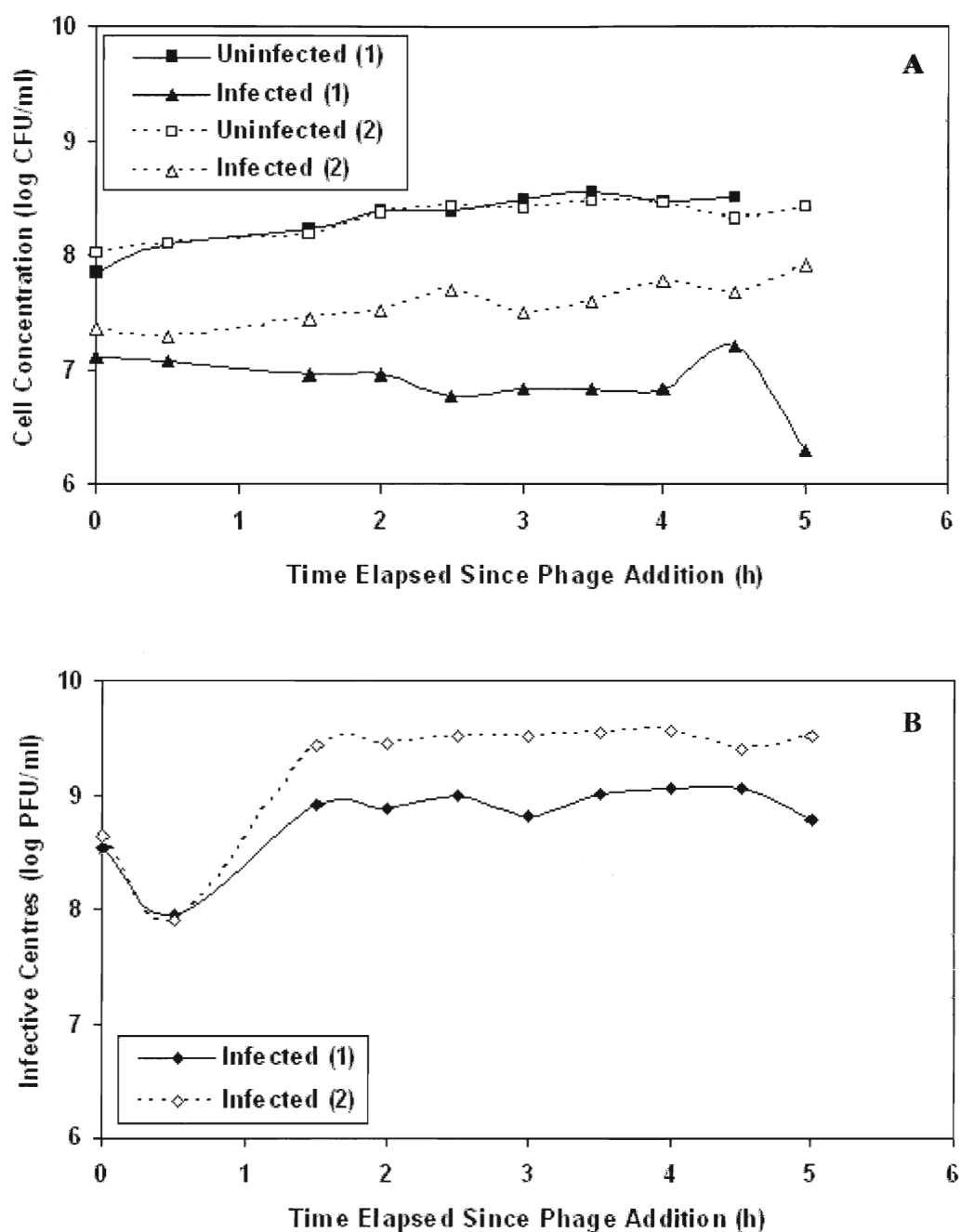


Figure 5-3. Growth of A) *E. amylovora* Ea29-7 and B)  $\phi$ Ea31-3 in synchronously infected and uninfected control cultures. Data from two independent trials are shown (open vs. closed points).

uninfected culture. In trial 1, the overall growth of the infected culture essentially mirrored that of the uninfected one, although at a lower concentration. In trial 2, the cell concentration in the infected culture did not change substantially over the course of the experiment, until showing a sharp rise and drop at the 4.5 - 5.0 h point. The total number of infective centres, meaning free phages and infected cells, declined over the first 30 min after phage were added to the culture, and then rose sharply at 1.5 h. Another drop and spike in the concentration of infective centres was observed after 4 h, and may indicate a second lysis event.

Experiments conducted with  $\phi$ Ea35-4 and Ea110 at 23°C showed similar trends, with lysis beginning about 1.5 h after phage inoculation, and phage concentration reaching a maximum 2 h after phage inoculation (data not shown).

#### *Phage Survival in Liquid Media*

Phages may be exposed to different liquid media during the production and processing of lysates for field trials. Table 5-2 gives the rates of change in the concentration of viable  $\phi$ Ea46-1A2 in six of these media over a 38 wk period. During the first 11 wk, the concentration of viable phages declined significantly in all media except autoclaved reverse osmosis water. In nutrient broth, this initial decline appears to have occurred in the first 4 wk. Between 13 and 38 wk, there was no significant decline in the concentration of viable phages in either nutrient broth ( $P = 0.252$ ) or autoclaved reverse osmosis water ( $P = 0.074$ ). Phage concentration continued to decline in all other media. The total decline in absolute phage concentration, adjusted for the 10-fold dilution at week 13, was no greater than 40-fold in any medium, and was within 10-fold for all media except the two involving tap water.

The survival of  $\phi$ Ea31-3, a small, short-tailed phage and  $\phi$ Ea46-1A2, a large, long-tailed phage were also tested in solutions of different ionic strengths. Table 5-3 gives the average rates at which the viable concentration of these phages changed in each solution over an 11 week period.  $\phi$ Ea31-1 was stable in sodium chloride solutions ranging from 0.015 to 0.5 M. The concentration of viable  $\phi$ Ea46-1A2 was only stable in 0.015 M NaCl, and declined significantly in the two solutions with greater ionic strength.

#### *Optimization of Phage-Carrier Application to Blossoms*

Four phages were used in blossom assays to test the effects of treatment timing and phage:carrier ratio (Figure 5-4). Thirteen phage-carrier combinations significantly reduced the severity of fire blight symptoms ( $P < 0.05$ ). When pathogen was applied immediately after treatment, all treatments significantly reduced disease severity relative to the control ( $P < 0.05$ ), but the efficacy of phage-carrier combinations was not significantly different from the efficacy of the carrier alone ( $P > 0.05$ ). When 3 h elapsed between treatment and pathogen application, the efficacy of phage-based treatments was greater than that of the carrier alone ( $P < 0.05$ ). The ratio of phage:carrier had no effect on disease severity ( $P > 0.05$ ).

Table 5-2. Survival of  $\phi$ Ea46-1A2 in various liquid media.

Solution	Rate of Change in Viable Titre ( $\log_{10}(\text{PFU/mL})/\text{wk}$ ) <sup>a</sup>	
	First 11 Weeks	13 to 38 Weeks
Autoclaved		
Nutrient Broth	- 0.033 *	- 0.002
PB with salts	- 0.048 *	- 0.014 *
Reverse Osmosis Water	- 0.072 *	- 0.010 *
Tap Water	- 0.044 *	- 0.038 *
Non-autoclaved		
Reverse Osmosis Water	- 0.00079	- 0.004
Tap Water	- 0.038 *	- 0.005 *

<sup>a</sup> Rates of concentration change marked with an asterisk are significantly greater than zero ( $P \leq 0.010$ ). Rates without an asterisk are not significantly greater than zero ( $P > 0.05$ ).



Table 5-3. Effect of ionic strength on the survival of  $\phi$ Ea31-3 and  $\phi$ Ea46-1A2.

Solution	Rate of Change in Viable Titre ( $\log_{10}(\text{PFU/mL})/\text{d}$ ) <sup>a</sup>	
	$\phi$ Ea31-3	$\phi$ Ea46-1A2
0.5 M NaCl	0.0028	-0.0042 *
0.15 M NaCl	-0.0001	-0.0033 *
0.015 M NaCl	0.0042	0.0036

<sup>a</sup> Rates of concentration change marked with an asterisk are significantly different from zero ( $P < 0.05$ ). Rates of change without an asterisk are not significantly different from zero.

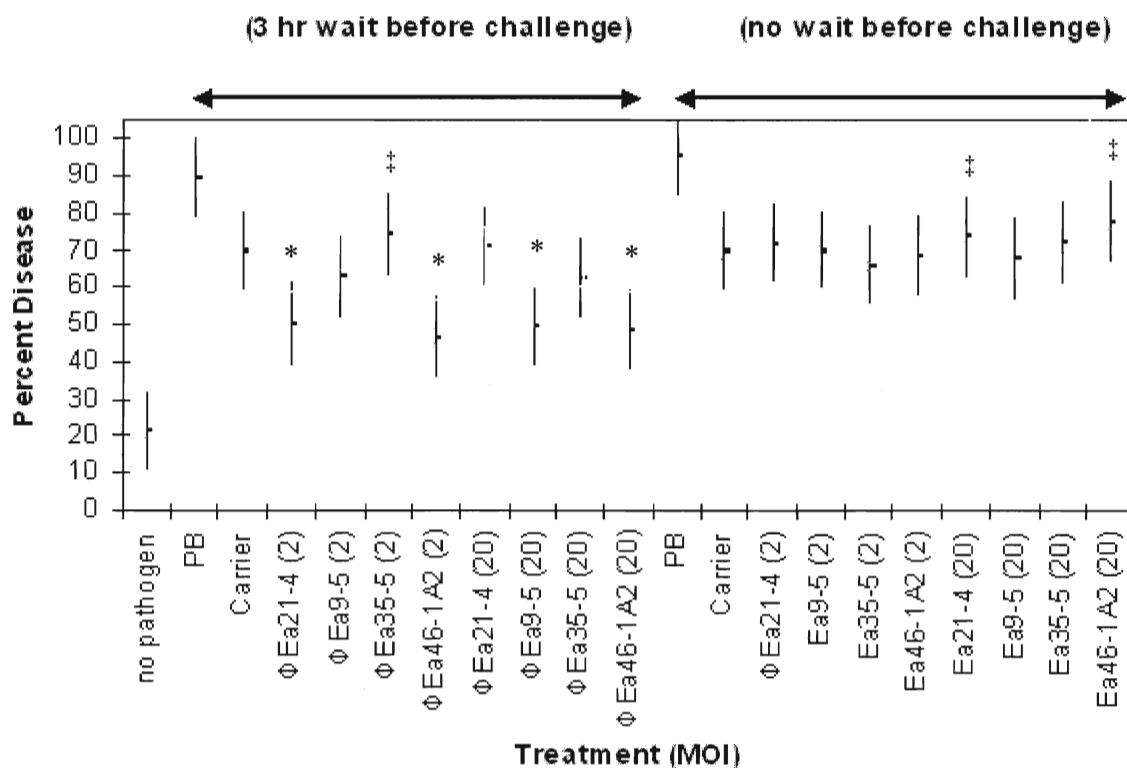


Figure 5-4. Effect of phage-carrier combinations on disease severity in blossom assays, mean  $\pm$  95% confidence limits for three replications (10 blossoms per replication). Four phages were applied with the carrier using an MOI of 2, and then again using an MOI of 20. These eight phage treatments, along with the carrier and an untreated control, were repeated twice: once, where 3 h elapsed between treatment and pathogen application (left-hand side), and once, where the pathogen was applied immediately after treatment (right-hand side). All phage and carrier treatments except those marked with ‡ resulted in significantly less severe disease than the PB control. Phage-carrier treatments marked with an asterisk were significantly better than the carrier alone.

## Discussion

Efficient propagation of phages and bacteria for *in planta* and field trials depends on their growth characteristics in liquid culture, at the approximate optimum temperature for *E. amylovora* growth. At 27°C, the growth of *E. amylovora* and *P. agglomerans* was quite similar.

The exponential doubling time of *E. amylovora* Ea6-4 was three times longer when the cultures were incubated in the water bath than when they were grown on the orbital shaker. Also, the doubling times of the two different *E. amylovora* strains grown using the water bath incubation were much less similar to each other than were the doubling times of the two different species studied using the orbital shaker. These differences were likely the consequence of differential oxygen availability, since the agitation of the orbital shaker was constant and sufficient to break the surface tension of the culture, whereas the flasks in the water bath were only shaken vigorously when samples were taken. Since equipment availability dictated the use of the water bath shaker for the subsequent phage growth experiments, that procedure was modified to include regular manual agitation of the culture flasks.

Regardless of the different rates of bacterial growth in each experiment, exponential growth had begun within 4 h of inoculation. Many phages require exponentially growing cells for their replication (Carlson, 2005), therefore phages being grown in large-scale liquid cultures for field use will be added to host cultures 4 h after the media is inoculated with the bacterial host. The rate and efficiency of phage adsorption can also be affected by changes in the expression of surface molecules due to the physiological state of the host (Guttman, Raya, & Kutter, 2005). Therefore aeration is likely to be critical for the efficient propagation of phages in liquid culture.

The latent period of  $\phi$ Ea31-3 in Ea110 is between 60 and 90 min, which is longer than

the 45 min latent period reported for the related phage,  $\phi$ Ea1 (Ritchie & Klos, 1979). The range in the estimated duration of latency is due to the apparently slow rate of phage adsorption. Phage concentration peaked 1.5 h after the addition of phage to the bacterial culture, but irreversible adsorption does not appear to have been complete until 30 min after phage were added to the culture. At time 0, immediately after addition of the phage, the concentration of bacteria in the infected culture was about 5-fold lower than the uninfected control. This suggests that approximately 80% of the cells were rapidly infected by at least one phage, and thus did not form colonies when plated on nutrient agar. Traditional Poisson models and empirical infection models would have predicted that an MOI of 10, with  $1 \times 10^8$  CFU/mL of susceptible host cells, would result in infection of 99.99% of those cells (Kasman et al., 2002). However, some culture conditions and certain phages are prone to lower than expected infection efficiencies (Carlson, 2005), and certain phage-host combinations can sometimes yield few productive initial infections while producing a first generation of progeny phage with very high infectivity (Evans, 1940; Wollman & Stent, 1952).

The additional manual agitation of these cultures compared to the previous cultures grown in the water bath did have an effect on bacterial growth. Though a different strain of *E. amylovora* was used in the phage growth experiments, the initial doubling time of cells in the uninfected Ea110 culture was shorter than it was for either of the strains in the second bacterial growth curve experiment. As in the earlier experiments, the growth rate of the uninfected bacterial culture slowed within 3 h of phage addition, or about 9 h after the culture was initiated.

Encapsulation within a polysaccharide matrix may be one way to preserve the phages for commercial use. These matrices are gelled at a moderate salt concentration and dissolved at a

higher one. Therefore the survival of two phages with different morphologies was monitored in solutions of varying ionic strength. The most dilute solution, 0.015 M, is similar to the ionic strength of the PB used throughout this research. The 0.15 M solution is standard physiological saline, and is similar to the concentration of sodium chloride that is used in PB<sub>salt</sub> for *Erwinia* phages (Gill, 2000; Ravensdale, 2004; this work). The 0.5 M solution is the highest concentration that is expected to be used to dissolve a polysaccharide encapsulation matrix. The viability of  $\phi$ Ea31-3 was unchanged after 11 wk in any of these solutions, but the viable titre of  $\phi$ Ea46-1A2 declined significantly in 0.15 M and 0.5 M solutions.  $\phi$ Ea31-3 is a member of the *Podoviridae*, and  $\phi$ Ea46-1A2 is a member of the *Myoviridae*. Based on only two phages, it is not clear whether their differential survival in saline is correlated with their morphologies, but clearly some phages are affected by storage at high salt concentrations.

Phages produced for use in the field will be grown in nutrient broth in order to nourish the host cells. However, the majority of the nutrient broth must be replaced with a non-nutritive buffer before use so as not to amend the nutritional status of the blossom in a way that would favour pathogen growth. Diafiltration will be used to concentrate the phage suspensions and filter out small dissolved substances, but the replacement buffer must be one in which the phages remain stable for an extended period of time. Other than nutrient broth, the only dilution medium in which the concentration of viable phage did not decline significantly was the autoclaved RO water. Inorganic chlorine in municipal tap water is known to decrease viral survival (Engelbrecht et al., 1980; Berg, Sanjaghsaz, & Wangwongwatana, 1989), as is the presence of microflora (Ward, Knowlton, & Winston, 1986). The reason for phage inactivation in PB<sub>salt</sub> may be the concentration of sodium chloride. This buffer contained 0.1 M sodium chloride, which is only

slightly less concentrated than the 0.15 M solution in which the viable titre of  $\phi$ Ea46-1A2 also declined. Both solutions were prepared with sterile reverse osmosis water.

These results are similar to results obtained for phages of *Erwinia carotovora* subsp. *carotovora*, where the concentration of viable phages was stable in fertilizer solutions made with autoclaved reverse osmosis water or  $PB_{salt}$ , but was unstable in solutions made with non-sterile water (tap or reverse osmosis) or with sterile tap water (Ravensdale, 2004). Tailed *E. coli* phage  $f_2$  also does not survive well when suspended in non-autoclaved distilled water (Lefler & Kott, 1974). However, the stability of  $\phi$ Ea46-1A2 in autoclaved reverse osmosis water and of  $\phi$ Ea31-3 in sodium chloride solutions was unexpected, since divalent metal cations are often required to maintain viability (Adams, 1959; Gill, 2000; Ravensdale, 2004), and none were added to these media. Either these phages are stable in their absence, or the ions were present in trace amounts in the reverse osmosis water, as the lab supply of this water is not completely deionized. The stability of  $\phi$ Ea46-1A2 in dilute sodium chloride also appears to contradict the results of the tap water tests, in which the decline of the  $\phi$ Ea46-1A2 population was attributed to the presence of chlorine species. However, where the dissolution of sodium chloride salt releases chloride ions, the dissolution of highly acidic chlorine gas or highly basic hypochlorite in municipal drinking water produces mostly hypochlorous acid (HOCl) and hypochlorite (OCl<sup>-</sup>) at pH values greater than 3.0 (Edstrom, 2003). HOCl, in particular, is a strong oxidizing disinfectant.

Once phages are produced and delivered to the blossom, their efficacy depends on their interactions with the *P. agglomerans* carrier and the *E. amylovora* pathogen. The results of the blossom assay show that the carrier can reduce symptom severity on its own, but that the addition of certain phages can increase that efficacy. The greatest efficacy was achieved when the phage-

carrier combination had time to become established on the blossom surface prior to the arrival of the pathogen. This is consistent with field studies in which suppression on *E. amylovora* populations was directly associated with early establishment of BlightBan A506 and C9-1 (Johnson et al., 1993b; Nuclio et al., 1998). If early phage-host interactions on the blossom, while both are still suspended in the applied liquid droplets, resemble phage growth in liquid culture, then the elapsed 3 h would have easily allowed one generation of progeny phage to be released from the initial carrier population. The uninfected carrier cells would have had a 3 h advantage over the arriving pathogen population in terms of adapting to the new nutritional environment and beginning to replicate.

There was no significant effect of MOI in this experiment, so this should be tested in field trials. Some effect of MOI is to be expected, since a very small population of phages may not replicate quickly enough to significantly impact pathogen growth, but the carrier population may be rapidly lysed if the MOI is too high. A dynamic equilibrium between phage and carrier is necessary in order to maintain the biopesticide on the blossom surface for several days. With this in mind, and since there was no clear effect of MOI in the blossom assays, field trials will begin with an MOI of 1.

Based on these experiments, the following parameters will be used for field trials: when phages are grown in large-scale liquid culture, phages will be added to the bacterial cultures 4 h after their inoculation, at the start of exponential growth; liquid cultures of phages must be very well aerated; phage-carrier treatments will be applied well before the pathogen, so that they can become established on the blossoms; phage-carrier combinations will be mixed at least 30 min prior to application, in order to ensure that infected carrier cells are applied to the blossoms.

## Chapter 6: Monitoring *Erwinia* phage populations in orchard soil

### Abstract

In order to develop a biopesticide for practical use, the environmental fate of its components must be monitored in the orchard soil. However, the direct quantification of microorganisms that have been washed from soil is complicated by the presence of soil-derived PCR inhibitors. Five elution media were tested, all at pH 8.0: nutrient broth, nutrient broth with 0.1% egg albumin, 250 mM glycine, 10 mM tetrasodium pyrophosphate, and 10 mM PB<sub>salt</sub>. All media were equally efficient at eluting phages from samples of sandy soil, but glycine and nutrient broth permitted the most sensitive detection of phages by real-time PCR. In all cases, PCR detection was not possible immediately following the elution of phages from soil. The addition of 0.1 M EDTA, followed by diafiltration to remove the EDTA, was the most effective means of removing soil-derived PCR inhibitors from the phage-containing eluate. Even 600-fold dilution of soluble substances in the eluate was less effective than the combination of chelation and diafiltration. This suggests that metals were the principle means of PCR inhibition. This method of removing PCR inhibitors without DNA extraction is best suited for processing a small to moderate number of large samples.



## Introduction

A critical component of biopesticide registration requires accounting for the environmental fate of its components, ideally in a quantitative fashion. Traditionally, biological agents are enriched prior to detection. If even one viable microbial cell or infective phage is present in a sample, it should be detectable following appropriate enrichment. In reality, the efficiency of enrichment can be greatly affected by the enrichment conditions. Viable bacterial cells can be rendered non-cultivable by certain environmental stresses (Wilson & Lindow, 2000), infective phages may be temporarily unavailable to host bacteria as a result of interactions with charged substances, and even the recovery of available phages is affected by the type and number of their specific hosts (Jensen et al., 1998). However, the sensitivity afforded by enrichment comes at the cost of being able to quantify the initial population.

Quantification requires that the target be detected without altering the population size, either directly within the sample environment or after extracting the target organism from that environment. DNA-based methods are generally the most sensitive, but are often inhibited by substances in the sample environment. Chapters 2 and 7 of this work describe the monitoring of bacteria and phage on aerial plant tissues, but the phage-carrier biopesticide is also expected to be washed into the soil surrounding the treated trees as a result of rain. New techniques are therefore required in order to easily track the persistence of *Erwinia* phages in soil.

The survival of *Erwinia* phages in soil has not been studied extensively. *E. amylovora* phages are easily isolated from soil beneath rosaceous hosts exhibiting signs of an active fire blight infection (Erskine, 1973; Schnabel & Jones, 2001; Gill et al., 2003), but have not been recovered from soil beneath healthy trees, even using enrichment techniques (Ritchie & Klos,

1977; Schnabel & Jones, 2001; A. M. Svircev, personal communication). Similarly, free *Bacillus* phages were recovered from environmental soil samples in very low numbers until the population of endogenous bacterial hosts was increased by adding rich media and incubating the resultant slurry at 37°C (Tan & Reanney, 1976). In the presence of actively growing host cells, high concentrations of multiple phage strains were recovered. These results suggest that long-term phage survival in soil is limited, but that a small number of phages may often persist, constituting a parent population that can quickly increase in number under favourable conditions.

Direct quantification of phages from soil generally depends on microscopy or on specific serological detection such as ELISA (Shigeharu et al., 2000; Williamson, Radosevich, & Wommack, 2005). Unfortunately, microscopic methods are time-consuming, and serological methods are semi-quantitative and are less sensitive than DNA-based techniques. Ashelford et al (2003) used TEM to count phages in filtered soil suspensions, and reported populations at least 350-fold higher than those estimated from viable plaque counts. However, the ecological significance of these extra phage is questionable since some of these samples did not yield any viable phages even after enrichment on multiple hosts (Ashelford, Day, & Fry, 2003).

Recovery of those limited numbers of surviving phages from soil is also hindered by adsorption of phages to soil particles (Bitton, 1975; Burge & Enkiri, 1978). Adsorption is mediated by pH-dependent electrostatic interactions (Burge & Enkiri, 1978; Taylor, Moore, & Sturman, 1981; Dowd et al., 1998) and seems to be greater for phages with longer tails (Ashelford, Day, & Fry, 2003; Williamson, Wommack, & Radosevich, 2003). Several detailed studies of elution techniques have been published (Lanning & Williams, 1982; Hu, 1998; Williamson, Wommack, & Radosevich, 2003), but such studies often lack the type of truly

systematic approach that is needed to ascertain which factors influence the success or failure of a particular method. Hu (1998) recovered more coliphages with glycine buffer than with beef extract, but the difference may have due to differences in the sample treatment associated with each medium, rather than the innate characteristics of the two media. He did find that phage recovery improved with increasing contact time between 30 and 90 min. Williamson, Wommack, and Radosevich (2003) tested the recovery of five different phages from two soil types using 10% beef extract, 1% potassium citrate, 10 mM sodium pyrophosphate, and 250 mM glycine. Phage recovery was greatest with beef extract and glycine, but recovery varied significantly between phage and soil types. Additionally, the beef extract and glycine were the only media prepared to a non-neutral pH (pH 9.0 and 8.0, respectively, compared to pH 7.0), which could be the real reason for their success.

The most useful study of phage elution was conducted by Lanning and Williams (1982). In order to recover actinophages from multiple soil types they compared the efficacy of nutrient broth, 3% beef extract, 1 M glycine, 1 M sodium nitrate, peptone/yeast/calcium broth, and glucose/Casamino acid/proline broth, all at the same pH. After obtaining the best results with nutrient broth they then compared recovery by nutrient broth at different pH levels, and with different types of exogenous protein. Recovery was most efficient using nutrient broth at a pH of 8.0, and only egg albumin improved phage recovery. Despite the variation in methodology among these studies, three common themes emerge: the efficacy of any given treatment varies with soil type, is lower for phages with long tails, and tends to be better for eluants at slightly basic pHs.

Successful elution methods do not necessarily equate to successful quantification since soil contains many substances that are known to inhibit PCR, and that tend to be washed from the soil along with the recovered phages. As little as 10 ng humic acid can inhibit a conventional, endpoint PCR reaction (Tsai & Olsen, 1992). It has been postulated that phenolic moieties in humic substances react with, and covalently bind to, DNA and protein, preventing the necessary interactions between the polymerase and the target DNA, or between primers and target DNA (Young et al., 1993). Humic acids may also interfere with the fluorescence processes upon which real-time PCR depends by quenching fluorescence of SYBR Green, Hoescht 33258, and PicoGreen complexed to DNA (Bachoon, Otero, & Hodson, 2001; Zipper et al., 2003). In the case of SYBR Green, which has excitation and emission wavelengths in the same range as the dyes used for probe-based real-time PCR, this is due to multiple factors: absorption of radiation at the fluorophore excitation and emission wavelengths by humic acid; diffusive collisions between humic acid and the fluorophore; and the formation of stable complexes between certain humic acids and both bound and unbound dye (Zipper et al., 2003).

A variety of metal ions can inhibit PCR, apparently by interfering with the binding and activity of the polymerase enzyme. Calcium ions in milk can interfere with PCR amplification (Bickley et al., 1996). Iron and other heavy metals are generally present in soil, whether in high levels as pollutants, or levels appropriate for micronutrition of plants, and are known PCR inhibitors (Wilson, 1997a; Ogram, 1998; Hao, Dick, & Tuovinen, 2002). Any substances that sequester  $Mg^{2+}$  will also inhibit the polymerase enzyme, which requires the ion as a cofactor and is sensitive to changes in its concentration (Satsangi et al., 1994; Wilson, 1997a). Polyamines (Ahokas & Erkkila, 1993), phenol (Katcher & Schwartz, 1994), and plant polysaccharides

(Demeke & Adams, 1992) can also inhibit amplification by directly affecting the DNA polymerase. Foulds et al (2002) were able to remove PCR inhibitors by washing *E. coli* cells collected from environmental water samples with EDTA, a metal ion chelator, prior to DNA extraction. Extensive work has also been done to develop methods of removing these inhibitors in the course of extracting total community DNA from soil or soil eluates (Zhou, Bruns, & Tiedje, 1996; Sjöstedt et al., 1997; Miller et al., 1999; Desai & Madamwar, 2006).

The aim of this work is to develop a rapid, simple method of monitoring the persistence of *Erwinia* phages in the soils of orchards where the phage-based biopesticide has been used. Unlike most other studies, soil samples were not homogenized, dried, and sieved prior to use. It is unlikely that actual field samples would receive this kind of treatment, since it might damage the phages trying to be recovered. Instead, large clumps of soil were broken up when the soil samples were weighed out, and when the flasks were shaken to mix the soil with the phage inoculum. Five elution media were tested in an attempt to optimize the recovery of phage particles from soil samples. These media were chosen from those that produced the best results in previous studies (Lanning and Williams, 1982; Hu, 1998; Williamson, Wommack, & Radosevich 2003). All elution media were adjusted to a pH of 8.0, since those studies also indicate that buffers having a higher pH are more likely to disrupt the electrostatic interactions between phage and soil particles. The elution methodology is based on the results of Lanning and Williams (1982) as they conducted the most extensive assessment of the effects of contact time and motion on phage recovery.

Several methods of removing soil-derived PCR inhibitors from the eluate were tested, in particular the use of a pre-amplification EDTA treatment. Since EDTA also chelates  $Mg^{2+}$ , it is,

itself, a PCR inhibitor. EDTA was therefore removed by diafiltration, a process that allows undesirable small solutes to be removed from a suspension medium during pressure-driven filtration, without diluting the larger substance of interest (in this case, the phage). During diafiltration, the dilution effects of successive additions of clean media are multiplied, allowing high dilution rates to be achieved with comparatively small volumes of fresh media.

## Methods

### *Growth Media and Strains*

Two bacteria and one bacteriophage were used in this work: *E. amylovora* Ea6-4, *P. agglomerans* Eh21-5, and *E. amylovora* phage  $\Phi$ Ea45-1B. All strain origins are described in Tables 2-1 and 2-2.  $\Phi$ Ea45-1B was grown on *E. amylovora* Ea29-7 in overnight liquid cultures using 8 g/L Difco nutrient broth. Lysates were treated with 2% (v/v) chloroform for 30 min, centrifuged at 8 500 xg for 25 min, and syringe- filtered using 25 mm diameter, 0.2  $\mu$ m, surfactant-free cellulose acetate filters (Nalgene, Rochester, NY). Filtered lysates were stored in nutrient broth at 4°C. Bacteria were grown overnight at 28°C on 23 g/L Difco nutrient agar.

### *Soil Sample Preparation and Analyses*

Several 30 cm x 2 cm soil cores were collected from the root zones of Bartlett pear trees in experimental orchards at the Agriculture and Agri-Food Canada (AAFC) research farm in Delhi, Ontario. Pooled soil cores were stored at 4°C between collection and use. Soil analyses had been previously conducted by A & L Laboratories (London, ON) in 2004 and 2005. The results of these analyses were obtained from Barry Kemp, the Farm Manager for AAFC-Vineland (encompassing both the Jordan and Delhi farms).

Experimental soil samples consisted of 10 g (wet weight) of soil in 250 mL capped Erlenmeyer flasks. Samples were sterilized by autoclaving the flasks for 20 min at 121°C, under steam pressure. Soil samples were spiked with 1 mL of  $1 \times 10^8$  PFU/mL of  $\Phi$ Ea45-1B, and mixed by shaking. Negative controls were inoculated with 1 mL of sterile nutrient broth. Inoculated soil samples were stored overnight at 4°C.

### *Phage Elution*

Five solutions were used to elute the phages: nutrient broth (pH 8.0), nutrient broth (pH 8.0) containing 0.1% (w/v) grade II egg albumin, 250 mM glycine (pH 8.0), 10 mM tetrasodium pyrophosphate (pH 8.0), and 10 mM  $\text{Pbsalt}$  (pH 8.0).

To elute the phages from the soil sample, 5 mL of solution was added to the flask and incubated for 25 min at 4°C. The flask was then placed on an orbital shaker at 200 rpm for 30 min at 4°C. The entire contents of the flask was transferred to 50 mL round-bottom centrifuge tube and centrifuged at 8 000  $\times g$  for 10 min at 4°C. The supernatant was decanted into a sterile tube for storage at 4°C. The sedimented soil was resuspended in 10 mL of the same solution and returned to the orbital shaker for another 30 min, then decanted and centrifuged as before. The first and second supernatants were combined and were stored at 4°C. This procedure was conducted with each solution, for both an inoculated soil sample and an uninoculated, phage-free, control sample. The titre of each eluate was determined using the soft agar overlay method described in Chapter 2.

The entire process was repeated twice at a later date, with the following change: only the supernatant from the first elution was transferred from the Erlenmeyer flask to the centrifuge tubes. The remaining solids were returned to the shaker with the second volume of elution media while the first batch was being centrifuged. Again, the first and second eluates from each flask were combined and stored at 4°C. Data were analyzed with SASv8.2 (SAS Institute, Cary, NC) using the general linear model (PROC GLM), with the Tukey adjustment for multiple comparisons.



### *Real-time PCR*

TaqMan-style real-time PCR reactions were conducted using primers and probes developed by Dr. W. -S. Kim (unpublished). Each reaction was conducted in a total volume of 25  $\mu\text{L}$ , and contained 1X Brilliant QPCR Master Mix (Stratagene, La Jolla, CA), 200  $\mu\text{M}$  of each primer, 100  $\mu\text{M}$  probe. Reactions were run in a Stratagene Mx4000 Multiplex Quantitative PCR system (Stratagene) under the following conditions: 95°C for 10 min; 40 cycles of 95°C for 30 s and 60°C for 60 s, with three endpoint fluorescence readings during each amplification segment.

A standard curve was constructed to allow quantification of  $\Phi\text{Ea45-1B}$  in soil eluates. Templates for the standard curve were prepared by serially diluting the same phage suspension used for soil inoculations in nutrient broth. Five microliters of each dilution was used as the reaction template. PCR reactions were run in duplicate

The efficiency of phage elution from inoculated soil samples was tested using 5  $\mu\text{L}$  of each eluate as a template. Fresh media of each type, containing  $3 \times 10^8$  PFU/mL of phage, were used as positive controls. Sterile reverse osmosis water was used as a negative control.

To confirm the presence of soil-derived PCR inhibitors in the eluted phage suspensions, reactions were conducted using the following as templates: 1  $\mu\text{L}$  of a phage suspension in fresh nutrient broth (pH 8.0), 1  $\mu\text{L}$  of a phage suspension in nutrient broth that had been passed through sterile soil, 5  $\mu\text{L}$  of a phage suspension in nutrient broth passed through soil, or sterile water. Phage suspensions contained  $4.4 \times 10^7$  PFU/mL. Identical reactions were also run using *E. amylovora* Ea6-4 and *P. agglomerans* Eh21-5 in the same media.

All amplifications were conducted in 25  $\mu\text{L}$  volumes and were run in duplicate. Information on the primers and probes is given in Table 6-1. Phage detection was based on

release of the 6-carboxyfluorescein (FAM) fluorescent reporter from the 5' end of the hydrolyzed probe, *P. agglomerans* detection was based on release of hexachlorofluorescein (HEX), and *E. amylovora* detection was based on release of Cy5. Probes were synthesized by Integrated DNA Technologies (Coralville IA, USA), and were labelled at the 3' end with either Black Hole Quencher 1 (BHQ-1) or Iowa Black (IABRQ).

### *Removal of PCR Inhibitors*

Three treatments were tested in an effort to remove soil-derived PCR inhibitors from the phage eluted with nutrient broth at pH 8.0: centrifugation, DNA extraction, and chelation.

The centrifugation and chelation treatments were conducted using the same initial numbers of phage. For centrifugation, 5 mL of eluate was added to 25 mL of nutrient broth in a 50 mL FEP centrifugation tube, and centrifuged at 16 000 xg for 45 min, at 4°C. The additional volume of nutrient broth was required to meet the manufacturer's guidelines for high-speed use of the centrifuge tubes. Following centrifugation, the supernatant was immediately decanted, and the sedimented material was resuspended in 500 mL of clean nutrient broth, pH 8.0. Both fractions were stored at 4°C. The concentration of viable phages in each fraction was determined using the soft agar overlay method described in Chapter 2.

For chelation, EDTA was added to 5 mL of eluate for a final EDTA concentration of 0.1 M, and the mixture was incubated at room temperature for 20 min. One hundred fifty milliliters of clean nutrient broth, pH 8.0, was added in order to dilute the EDTA. The volume of the suspension was reduced to approximately 20 mL by normal flow diafiltration using an Amicon Model 8400 stirred cell apparatus (Millipore, Billerica, MA) with a 100 kDa YM-type

regenerated cellulose membrane filters. In order to further reduce the concentration of EDTA in the retained phage suspension, an additional 100 mL of clean nutrient broth was added to the phage suspension, and the retentate volume was again reduced to approximately 20 mL. The Amicon apparatus was assembled according to manufacturer's instructions. Preservatives were removed from new filters by floating them skin-side down for at least one hour, with three changes of water. Approximately 400 mL of distilled water was flushed through the system prior to each run. Phage suspensions were concentrated under 50 psi of nitrogen pressure until the retentate was reduced to the desired volume. Residual phages adhering to the filtration membrane were collected by floating the membrane upside down in a sterile petri dish containing a small volume of nutrient broth, and shaking the dish gently. Two such washes were added to the retained phage suspension. Both the retentate and filtrate were stored at 4°C. The concentration of viable phages in each fraction was determined using the soft agar overlay method described in Chapter 2. Filters were used no more than three times each. In between uses, filters were sterilized by floating them in 70% ethanol for 20 min, then in 0.1% TergAZyme for 30 min. Sterilized filters were flushed with distilled water and stored in 10% ethanol at 4°C.

A one-step DNA extraction was also tested. Five hundred microliters of 24:1 chloroform:isoamyl alcohol was added to an equal volume of eluate, and mixed by gentle inversion for 5 min. The emulsion was centrifuged at 13 000 xg for 5 min. The upper, aqueous layer was removed with a pipet and transferred to a sterile microcentrifuge tube. DNA was precipitated from a 200 µL aliquot of the crude extract by adding sodium acetate to a final concentration of 0.3 M, and an equal volume of 95% ethanol. The precipitated DNA was collected by centrifugation at 13 000 xg for 5 min, washed with 70% ethanol, and allowed to

airdry before being resuspended in 10 mM Tris-HCl. Both the precipitated DNA and the remaining crude extract were stored at 4°C.

### *Endpoint PCR*

Amplification of phage DNA from the treated eluates was attempted using the  $\phi$ -dpo1 primers (see Table 6-1). Amplification reactions were conducted in 25  $\mu$ L volumes. Each reaction contained 400  $\mu$ M of each primer, 200  $\mu$ M each of dATP, dCTP, dGTP, and dTTP, 1.5 mM MgCl<sub>2</sub>, 1.25 U *Taq* (MBI Fermentas), 1X polymerase buffer, and 2  $\mu$ L of phage suspension in nutrient broth. Reactions were run in an Applied Biosystems GeneAmp 9700 thermal cycler under the following conditions: 94°C for 5 min; 30 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 30 s; 94°C for 10 min. Reaction products were visualized using agarose gel electrophoresis, as described in Chapter 2.

### *Optimization of EDTA Treatment*

The relative contributions of chelation and solute dilution to the success of the EDTA-based treatment were determined by repeating that treatment with certain modifications. First, the process was repeated exactly, but using a final concentration of 0.01 M EDTA instead of 0.1 M. Second, the original treatment was repeated using sterile water in lieu of the EDTA solution. Third, five times the initial amount of eluate was used, again with no addition of EDTA. Finally, the original treatment was repeated using water in lieu of EDTA, but this time using twice the volume of diluent at each stage of diafiltration, such that 300 mL was reduced to 20 mL, and then diluted to 200 mL and again reduced to 20 mL.

Table 6-1. PCR primers (F or R) and probes (P) for *Erwinia* phages, *E. amylovora*, and *P. agglomerans*.

Oligonucleotide	Target Species <sup>a</sup>	Target Gene	Amplicon Size (bp)
Φ-dpo1F	ΦEa45-1B	depolymerase ( <i>dpo</i> )	171
Φ-dpo1R			
Φ-dpo1P			
Ea-lscF	<i>E. amylovora</i>	levansucrase ( <i>lsc</i> )	105
Ea-lscR			
Ea-lscP			
Pa-gndF	<i>P. agglomerans</i>	gluconate-6-dehydrogenase ( <i>gnd</i> )	73
Pa-gndR			
Pa-gndP			

<sup>a</sup> Design was based on the following sequences deposited in the NCBI nucleotide database: AJ278164 (*dpo*), X75079 (*lsc*), and AF208633 (*gnd*).

## Results

### *Chemical Characteristics of Orchard Soils*

Tables 6-2 and 6-3 show the results of soil analyses conducted on the experimental apple and pear orchards at the AAFC-Delhi site, and the AAFC-Jordan site. The Jordan site is located in the Niagara fruit growing region and is much more typical of commercial orchards than the Delhi site.

The Jordan soil contains more organic matter and therefore has a higher pH and cation exchange capacity (CEC). This greater ability to hold and supply cations is reflected in the higher levels of magnesium and calcium in the Jordan soil. The Delhi and Jordan soils appear to contain similar concentrations of iron and bicarbonate phosphorus, but the Delhi soil contains less zinc and copper, and more manganese.

### *Elution of Phages and the Presence of Soil-derived PCR Inhibitors*

The recovery of phage from soil was assessed in two ways: viable titre, and real-time PCR. Table 6-4 shows the percentage of the original phage inoculum that was recovered using each type of media. The relative efficiency of these media differed in each trial, but there was no significant difference in average recovery among the five media ( $P = 0.850$ ).

Figure 6-1 is the amplification plot from the real-time PCR assessment of phage recovery. Positive controls consisted of phage suspended in elution media that had not come in to contact with soil. Fluorescence signals were obtained from four out of five positive controls, but not from any of the soil eluates, suggesting the presence of a soil-derived PCR inhibitor in the experimental

Table 6-2. Chemical characteristics of orchard soil at the sampling sites on the AAFC-Delhi Research Farm. Data are from 2004.

Plot #	pH	CEC <sup>a</sup> (meq/ 100 g)	Elemental Concentration (ppm) <sup>b</sup>							P (bicarb)
			Mg	Ca	Al	Fe	Cu	Mn	Zn	
9	6.9	3.8	60 <sup>M</sup>	350 <sup>L</sup>	1080	98 <sup>VH</sup>	0.8 <sup>M</sup>	80 <sup>VH</sup>	3.8 <sup>M</sup>	66 <sup>VH</sup>
11	6.8	5.2	90 <sup>M</sup>	590 <sup>M</sup>	937	-	-	-	-	64 <sup>VH</sup>
22	6.6	4.6	75 <sup>M</sup>	500 <sup>L</sup>	846	-	-	-	-	58 <sup>VH</sup>

<sup>a</sup> Cation exchange capacity

<sup>b</sup> Superscripts indicate whether the micronutrient level is low (L), medium (M), high (H), or very high (VH), from the perspective of soil fertility.

Table 6-3. Chemical characteristics of typical orchard soil on the AAFC-Jordan Research Farm. Data are from 2005.

Plot #	pH	CEC <sup>a</sup> (meq/ 100 g)	Elemental Concentration (ppm) <sup>b</sup>							P (bicarb)
			Mg	Ca	Al	Fe	Cu	Mn	Zn	
2B	7.1	10.3	230 <sup>H</sup>	1370 <sup>H</sup>	-	88 <sup>VH</sup>	3.9 <sup>VH</sup>	47 <sup>M</sup>	10.4 <sup>H</sup>	56 <sup>VH</sup>
13	7.2	7.8	195 <sup>H</sup>	1020 <sup>H</sup>	-	99 <sup>VH</sup>	6.6 <sup>VH</sup>	30 <sup>M</sup>	5.6 <sup>M</sup>	40 <sup>H</sup>
21	7.3	8.9	140 <sup>H</sup>	1420 <sup>H</sup>	-	107 <sup>VH</sup>	3.8 <sup>VH</sup>	33 <sup>M</sup>	8.8 <sup>H</sup>	59 <sup>VH</sup>

<sup>a</sup> Cation exchange capacity

<sup>b</sup> Superscripts indicate whether the micronutrient level is low (L), medium (M), high (H), or very high (VH), from the perspective of soil fertility.

Table 6-4. Effect of elution medium on recovery of the original  $1 \times 10^8$  PFU. Data for trials 2 and 3 are the mean of three subsamples taken from each eluate.

Medium	Percent Recovery of Original Phage Inoculum			
	Trial 1	Trial 2	Trial 3	Mean $\pm$ SD <sup>a</sup>
Nutrient broth (pH 8.0)	31.5	37.1	43.4	37.3 $\pm$ 5.9 <sup>A</sup>
Nutrient broth (pH 8.0) + 0.1 % (w/v) egg albumin	23.9	42.1	44.7	36.9 $\pm$ 11.4 <sup>A</sup>
10 mM PB <sub>salt</sub> (pH 8.0)	16.4	46.2	30.9	31.1 $\pm$ 14.9 <sup>A</sup>
250 mM glycine (pH 8.0)	24.8	54.5	38.6	39.3 $\pm$ 14.9 <sup>A</sup>
10 mM tetrasodium pyrophosphate (pH 8.0)	24	39.5	30.1	31.2 $\pm$ 7.8 <sup>A</sup>

<sup>a</sup> Means with the same letter are not significantly different.



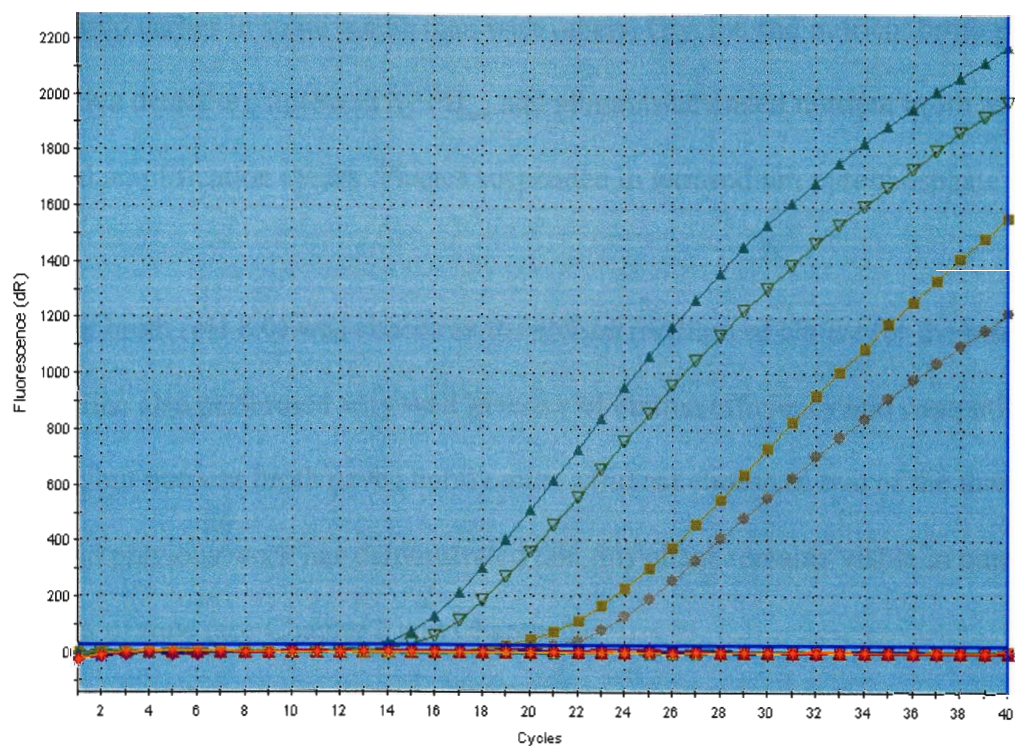


Figure 6-1. Effect of elution medium and passage through soil on the amplification of phage. Positive signals are from glycine (blue), nutrient broth (green), PB<sub>salt</sub> (gold), and nutrient broth with albumin (grey) positive controls.

samples. Furthermore, each positive control contained  $3 \times 10^5$  PFU per 25  $\mu\text{L}$  reaction, yet detection occurred earlier in some media than with others. Glycine and nutrient broth permitted the most sensitive detection, followed by  $\text{PB}_{\text{salt}}$  and albumin-amended nutrient broth after four or more additional amplification cycles. Phages suspended in tetrasodium pyrophosphate were not detected at all.

Nutrient broth (pH 8.0) was chosen as the elution medium of choice for the remainder of this work. Glycine also performed very well in terms of elution efficiency and compatibility with PCR detection, but nutrient broth gave slightly more efficient elution in two of the three trials (Figure 6-1) and previous work has demonstrated that this phage remains viable in nutrient broth for long periods of time (see Chapter 2 and Chapter 5).

The presence of soil-derived PCR inhibitors was confirmed by attempting to amplify the phage and two bacterial species, *E. amylovora* and *P. agglomerans*, from nutrient broth that had been passed through sterile soil according to the same procedure used to elute the phages. No fluorescence signals were obtained from any of the targets when they were suspended in the buffer that had been passed through soil, regardless of whether 1  $\mu\text{L}$  or 5  $\mu\text{L}$  of the template suspension was used. In contrast, signals were obtained from each target when it was suspended in clean nutrient broth.

### *Removal of PCR Inhibitors*

Three methods of removing the soil-derived PCR inhibitors from the eluate were tested. The eluted phage suspension was centrifuged to determine whether the inhibitory substances were associated with particulate matter in the suspension. A single-step organic extraction was

also performed in an attempt to obtain inhibitor-free DNA, and both the crude extract and the precipitated DNA were tested. In the third treatment, EDTA was added to the phage suspension in order to chelate metal ions. Since EDTA can also interfere with PCR, the EDTA was removed by diafiltration.

The distribution of viable phages in among the resulting fractions is shown in Table 6-5. Centrifugation allowed the collection of a more concentrated phage suspension, but only half of the phages present in the original sample remained viable, in either the supernatant or the resuspended pellet. Of the remaining viable phages, only slightly more than half were present in the sedimented fraction. Better recovery of viable phages was obtained with chelation and diafiltration. Fewer than 18% of viable phages were lost over the course of the treatment, with only 2.8% of the remaining phage being lost to the filtrate.

The efficacy of each treatment was evaluated by attempting to amplify a 171 bp segment of the phage depolymerase gene. Figure 6-2 shows the results of these amplification reactions. As expected, phage DNA was amplified from clean nutrient broth spiked with  $3.7 \times 10^7$  PFU/mL of phage, but not from nutrient broth that had been passed through soil. Substantial specific amplification was also achieved from the EDTA-treated sample, which contained  $1.9 \times 10^5$  PFU/mL. Very little amplification was obtained from the centrifugation supernatant, even though that fraction contained  $8.8 \times 10^4$  PFU/mL, which was only slightly less than the EDTA-treated sample. No phage DNA was amplified from any of the other treated fractions.

The relative contributions of chelation and dilution to the removal of PCR inhibitors were assessed by altering the amount of EDTA added and the degree of dilution, measuring the apparent phage concentration using real-time PCR, and comparing the efficiency of each

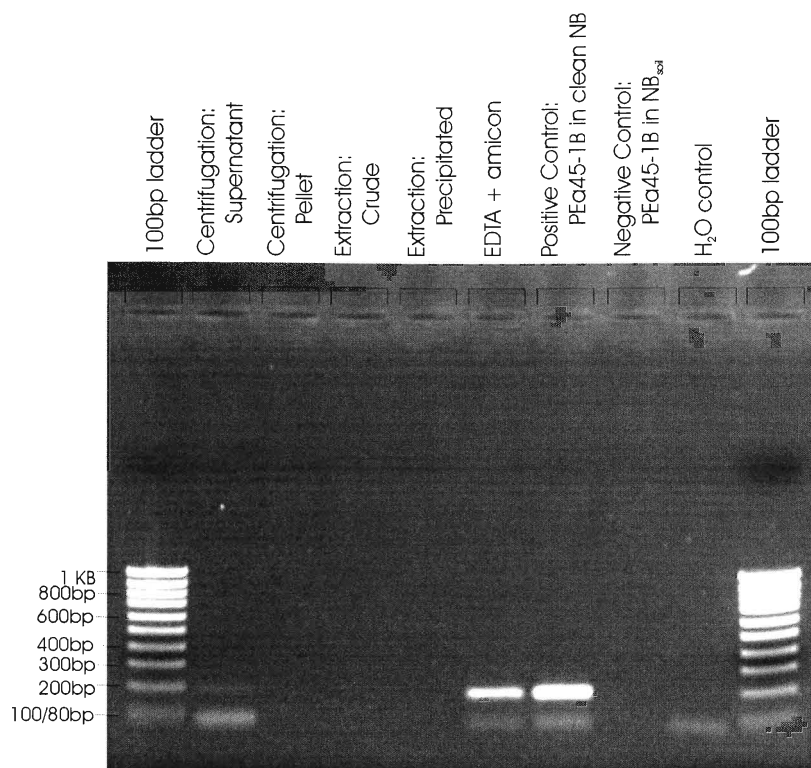


Figure 6-2. Amplification of phage DNA following treatment of soil eluate. Far left and right lanes contain GeneMark 100 bp DNA ladder (MBI Fermentas). The number of target gene copies present in each 25  $\mu$ L reaction was as follows:  $1.8 \times 10^2$ , centrifugation supernatant;  $1.3 \times 10^4$ , centrifugation pellet;  $4.2 \times 10^5$ , crude organic extract;  $4.2 \times 10^5$ , precipitated, extracted DNA;  $3.9 \times 10^2$ , diafiltration retentate; 2, diafiltration filtrate;  $7.4 \times 10^4$ , positive control;  $1.1 \times 10^5$ , negative (inhibition) control; 0, water control; these values are based on the viable titre of each fraction (Table 6-5), or, in the case of the organic extraction fractions, are inferred from the viable titre of the original soil eluate.

Table 6-5. Recovery of viable phages by centrifugation and diafiltration.

Treatment	Fraction	Viable Titre (PFU/mL)	Fraction Volume (mL)	Total PFU Present	% of Original Phages	
					Present	Lost
Centrifugation	Supernatant	$8.77 \times 10^4$	30	$2.63 \times 10^6$	25.1	44.6
	Pellet	$6.36 \times 10^6$	0.5	$3.18 \times 10^6$	30.3	
Chelation & Diafiltration	Retentate	$1.94 \times 10^5$	43	$8.36 \times 10^6$	79.6	17.6
	Filtrate	$1.02 \times 10^3$	238	$2.40 \times 10^5$	2.8	

treatment. The quantification of  $\phi$ Ea45-1B using real-time PCR was calibrated using serial dilutions containing a known concentration of viable phages. The standard curve is shown in Figure 6-3. The relationship between threshold cycle ( $C_T$ ) and initial quantity was linear across the tested 4-log range. The lower limit of phage detection was not explored in detail, but would not be expected to be much lower than the 80 PFU per 25  $\mu$ L reaction that was observed here, since this is on the same order of magnitude as the 20 CFU per reaction threshold described in Chapter 4.

Table 6-6 shows the relative efficiency of the different EDTA and diafiltration treatments. In the original treatment (No. 1), EDTA was added to an aliquot of the soil eluate to a final concentration of 0.1 M. By diluting that initial 5 mL sample to 150 mL and reducing the volume of retentate to 20 mL, the total concentration of dissolved EDTA would have been reduced by 30-fold. The subsequent dilution of that retentate to 100 mL resulted in a further 5-fold dilution, for a 150-fold dilution of solutes in total. The concentration of viable phage in the filtrate revealed a 3% loss across the 100 kD membrane. The expected concentrations of phage were therefore based on the concentration of the original sample and the volume used, less 3%. Under this expectation, treatment 1 removed most of the soil-derived PCR inhibitors, such that the estimate of phage concentration based on real-time PCR was 84% of the theoretical total. The same dilution rate, with a lower concentration of EDTA (treatment 2) was not nearly as effective. In the absence of EDTA, even very high dilution rates were less effective (treatments 3 and 5). Dilution alone was completely ineffective when the dilution rate was only a fifth of the original treatment (treatment 4).

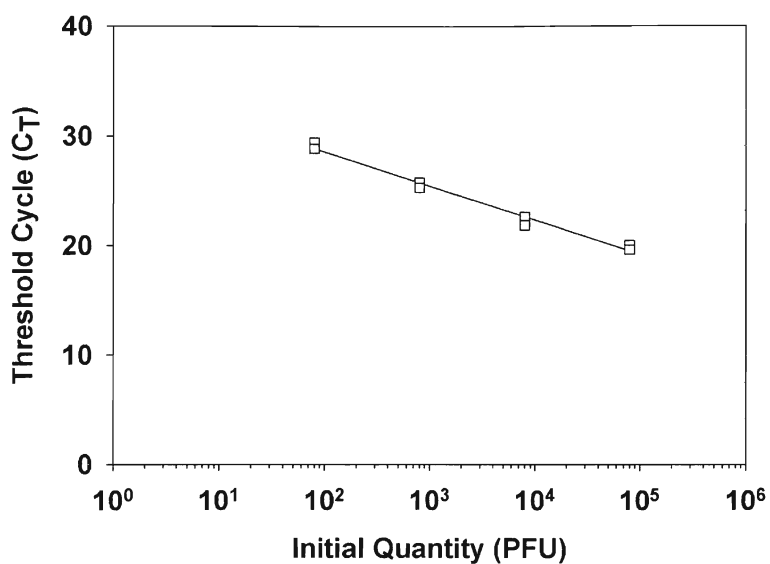


Figure 6-3. Standard curve for the real-time PCR amplification of  $\phi$ Ea45-1B using the  $\phi$ -dpo1 primers and probe. Initial quantities refer to the number of phages present in the 25  $\mu$ L PCR reaction. Quantification is given by the equation  $y = 34.7 - 3.11 \log(x)$ ;  $r^2 = 0.986$ .

Table 6-6. Relative contributions of chelation and dilution to the removal of soil-derived PCR inhibitors.

No.	Treatment			Phage Concentration According to real- time PCR (PFU/mL)	Expected Concentration (PFU/mL)	Efficiency of Treatment
	Volume of Eluate	EDTA	Dilution of Solutes			
1	5 mL	0.1 M	150-fold	$1.99 \times 10^5$	$2.37 \times 10^5$	84.0%
2	5 mL	0.01 M	150-fold	$2.70 \times 10^4$	$1.16 \times 10^5$	23.2%
3	5 mL	-	150-fold	$1.93 \times 10^4$	$1.33 \times 10^5$	15.0%
4	25 mL	-	30-fold	0	$6.98 \times 10^5$	0%
5	5 mL	-	600-fold	$4.01 \times 10^4$	$1.18 \times 10^5$	35.2%



## Discussion

There are many published studies describing DNA extraction techniques that can be used, with varying success, to remove soil-derived PCR inhibitors. The SDS-based method of Zhou, Bruns, & Tiedje (1996) was used successfully with several soil types, including sandy loams. The Ultraclean Soil DNA Isolation kit from MO BIO Laboratories has been used to extract total bacterial DNA for real-time PCR detection of *E. coli* O157:H7 (Ibekwe et al., 2002), though Desai and Madamwar (2006) describe a protocol that removes metallic and organic inhibitors more efficiently. Few studies bother to mention attempts to amplify microbial DNA directly from soil eluate, and those that do report consistent failure (Sjöstedt et al., 1997). Here, a method of removing soil-derived PCR inhibitors from soil eluates without DNA extraction is described.

There was no significant difference in the efficacy of the five tested eluents. Proteinaceous substances, and egg albumin in particular, have been found to aid the release of phages from soil (Lanning & Williams, 1982; Hurst et al., 1991), but no such improvement was observed here. Lanning & Williams (1982) did observe differences between the performance of different eluents at a given pH, but that was at pH 7.0, which was not the optimum value, at least for nutrient broth. The performance of the different eluents were not compared at the higher pH. Since adsorption is mediated by electrostatic processes (Taylor, Moore, & Sturman, 1981; Dowd et al., 1998), it is possible that there is no detectable difference between elution media at the optimum pH, but that at non-optimal pH levels, the specific types of charged species in each medium are more or less successful in disrupting the interactions between phages and soil particles. Since the primary goal of this work was to find a way to deal with PCR inhibitors, rather than to fully optimize the elution process, the elution media were only compared at pH 8.0.

The most important difference between the different eluents was their influence on the sensitivity of real-time PCR. When using fresh media, glycine offered the least interference with amplification and fluorescence detection. Phage detection in nutrient broth was only slightly less sensitive than in glycine. In a rich, undefined medium such as nutrient broth, any number of inhibitory substances could be present in trace amounts that would account for this difference. The PCR reaction will tolerate the presence of a certain amount of protein, as evidenced by the sensitivity of methods that use whole or boiled cells as templates (Starnbach, Falkow, & Tomkins, 1989; Salm & Geider, 2004; Chapter 4). Nevertheless, abundant protein can substantially reduce the sensitivity of PCR, presumably by non-specific blocking of DNA molecules (Rijpens et al., 1996). Thus it is not surprising that the presence of albumin reduced the sensitivity of real-time PCR. However, this inhibition is not a concern since albumin did not enhance the efficiency of phage elution.

Presumably, the reduced sensitivity of phage detection in  $PB_{\text{salt}}$  was due to the high salt concentration, even though the addition of 5  $\mu\text{L}$  of the buffer would only have increased the magnesium concentration in the reaction by 0.2 mM. The tetrasodium pyrophosphate buffer contained much less sodium than the  $PB_{\text{salt}}$ , but this compound is commonly used as a water softener, and likely inhibits PCR by sequestering  $\text{Mg}^{2+}$  ions. However, this implies that tetrasodium pyrophosphate would also help chelate soil-derived PCR inhibitors. It has been used by soil scientists to dissolve organic matter and extract metals bound to humic substances (McKeague, Brydon, & Miles, 1971; Manninen et al., 1996). It would be interesting to see whether phage elution with tetrasodium pyrophosphate, followed directly by diafiltration with a medium like nutrient broth, would remove soil-derived PCR inhibitors as effectively as the

EDTA-based treatment described here.

Regardless of the eluent used, soil-derived inhibitors prevented the direct quantification of both phages and bacteria in the soil eluate using real-time PCR. Several methods of eliminating these inhibitors were tested. The most successful treatment was clearly the combination of chelation and diafiltration, which suggests that metal ions are the main cause of PCR inhibition in these eluates. Calcium, iron, magnesium, copper, manganese, and zinc are present in appreciable amounts in the soil from which these samples were taken, as they are in most soils. All of these metals can exist as divalent cations, which are known to affect the efficiency of PCR (Satsangi et al., 1994; Bickley et al., 1996; Wilson, 1997a). Even elemental concentrations that are low from a nutritional standpoint can inhibit enzymatic reactions.

PCR amplification was not possible from most of the centrifugation, extraction, and chelation/diafiltration treatments. There was only a 2.8% loss of viable phages across the 100 kDa diafiltration membrane, which could be reduced by using a membrane with a smaller pore size. Given the small volume used for each PCR reaction, the number of phages in the filtrate was too low to be detected by PCR, regardless of whether inhibitors were present. In the absence of inhibitors, amplification should have been possible from all other fractions. The negative results from the organic extraction are consistent with other studies in which modified extraction protocols were needed to eliminate soil-derived PCR inhibitors. Single-step chloroform-isoamyl alcohol extraction has been as successful as multi-step phenol and chloroform-isoamyl alcohol extractions in removing PCR inhibitors from intraocular fluids (Wiedbrauk, Werner, & Drevon, 1995). However, the inhibitors present in the aqueous and vitreous fluids were not definitively identified, and may differ sufficiently in character from those present in soil.

Amplification from the centrifugation supernatant was much lower than from the EDTA-treated retentate, despite a very small difference between the phage concentrations in those two fractions. This indicates that a substantial amount of the inhibitor was still present in the supernatant, either complexed with the phage particles, or freely dissolved in the nutrient broth. Amplification was not possible from the sedimented fraction, which suggests that the inhibitor is mostly complexed with the sedimented particulate matter, though not necessarily to the phage particles themselves, as the soil eluate contained some small soil particles. Large losses of phages by adsorption to 0.2  $\mu\text{m}$  filtration membranes have generally not been observed during this work (see Chapter 7), but the soil eluate was not filtered in this experiment in order to guard against the potential effect of soil-derived substances on the electrostatic interactions between phages and the membrane.

Both centrifugation and diafiltration resulted in overall losses of viable phages. Presumably these phages were inactivated as a result of physical stress, or were bound to the membrane during diafiltration. Deformation of phages during high-speed centrifugation in the presence of particulate matter has been reported previously (Wommack et al., 1992; Ashelford, Day, & Fry, 2003), as has the adsorption of viruses to diafiltration membranes (Winona et al., 2001). In fact, tailed phages were particularly likely to be lost to membrane adsorption.

Since the quantity of phages estimated by real-time PCR estimate was 84% of the expected value, the differences in band intensity between the positive control and the EDTA-treated sample (Figure 6-2) is mostly attributable to the relative concentrations of phage in each of the tested fractions, rather than differential amplification efficiency. It should be noted that the actual concentration of viable phages in the retentate of treatment 1 was  $1.94 \times 10^5$  PFU/mL,

which is essentially almost identical to the concentration measured by real-time PCR. Viable phages in the original eluate sample were lost in the course of treatment, probably because of physical damage from the pressure in the filtration cell or by adsorption to the diafiltration membrane. However, since the exact route of loss is not known, and since physically damaged phages should still be detectable by PCR, the viable titre of the retentate does not necessarily reflect the true expected quantity, and treatment 1 can not conclusively be said to have completely eliminated PCR inhibitors. This question might be partially addressed by repeating the experiment with a phage from the *Podoviridae* family, since the compact morphology of *Podoviridae* may make them less susceptible than long-tailed *Myoviridae* like  $\phi$ Ea45-1B to inactivation by the physical forces imposed during diafiltration.

With a mean elution efficiency of 37% and a mean detection efficiency of 82%, the overall efficiency of this process was about 30%, which is well within an order of magnitude of the actual number of viable phages originally added to the soil samples. None of the other combinations of EDTA addition and diafiltration were as successful as the application of 0.1 M EDTA and 150-fold dilution (Table 6-6). Dilution alone resulted in the removal of some inhibitors, but was much less effective than the combination of chelation and filtration, even at a very high dilution rate. This suggests that divalent metal ions in the soil were a substantial cause of PCR inhibition.

The Delhi orchard soil is quite sandy, and so these results may not be directly transferable to richer soils that have greater cation exchange capacities and contain higher levels of humic acids. In addition, the method was developed using autoclaved soil so that the efficiency of phage elution and detection could be measured. This heat treatment may affect the presence of soil-

derived PCR inhibitors. Skipper and Westermann (1973) reported that autoclaving for 1.5 or 3.0 h increased soil pH by 0.2 units, and postulated that this was due to the breakdown of organic acids (Skipper & Westermann, 1973). A pH change that small should not impact the buffered PCR reaction mixture, but even though the sterilizing heat treatment used here was only 20 min, it is possible that certain PCR inhibitors could have been removed by the process. The efficacy of the chelation and diafiltration treatment should therefore be repeated on inoculated and uninoculated fresh soil.

The chelation and diafiltration method described here should be suitable for processing a small number of large samples, as in a metagenomic study of microbial communities in a particular environment. Unfortunately, the volumes and handling steps involved in diafiltration make it less appropriate for processing many small samples. If the phages applied as part of this biopesticide are rapidly inactivated in the soil, then large samples will have to be analyzed in order to recover any phages at all. In that case, this method has the advantage of permitting large volumes of soil eluate to be substantially concentrated during diafiltration.

If the use of many small samples is necessary, then a possible alternative option for removing the EDTA and chelated metals from the treated soil eluate might be to chemically precipitate the phages using polyethylene glycol. Prolonged incubation of phages with PEG 8000 can result in the precipitation of salts, but it may be possible to balance recovery of the phages with the elimination of EDTA. The use of *Tth* or *Tfl* polymerases should also be explored, as these have been shown to be much more resistant than *Taq* polymerase to inhibitors that directly affect the DNA polymerase (Katcher & Schwatz, 1994; Wiedbrauk, Werner, & Drevon, 1995). Failing that, DNA extraction from the soil itself should be attempted, rather than phage elution.

## Chapter 7: Successful field application of a phage-based biopesticide for fire blight

### Abstract

The efficacy of biopesticides for the control of *E. amylovora*, the causative agent of fire blight, were tested in field trials. In 2005, six of twelve treatments consisting of *E. amylovora* phages and a *P. agglomerans* “carrier” significantly reduced the incidence of blossom blight when tested using a randomized complete block design. The control afforded by these treatments was not statistically different from that afforded by streptomycin, which is the most effective treatment for the prevention of blossom blight. The population dynamics of the phage, carrier, and pathogen were monitored over the course of selected treatments. In treatments exhibiting a significantly reduced incidence of fire blight, the average blossom population of *E. amylovora* had been reduced to pre-experiment epiphytic levels. An average phage population greater than  $1 \times 10^5$  PFU/blossom at the time of pathogen arrival was required to significantly reduce the chance of *E. amylovora* infection.

## Introduction

“Unreliable” is one of the most damning criticisms that can be leveled at any therapeutic treatment, coming in right behind “the cure is worse than the disease”. Unreliability is the curse that has dogged phage therapy for almost 100 years. Leaving aside work done in the first half of the 20<sup>th</sup> century, before the biological and molecular nature of phages or genes were understood, the results are better, but there is still some ammunition for this charge. One issue is that the success of phage-mediated treatments depends on the survival and activity of a biological agent. Since biological control agents generally require a narrower range of environmental conditions than do chemical agents, their performance tends to be much more variable (Johnson & Stockwell, 2000; Johnson et al., 2000). However, the etiologic agents of disease also have specific environmental requirements, and since phage prey upon bacteria in natural ecosystems, their ecological niches must necessarily be similar. In this case, that ecological niche is the apple or pear blossom, and the prey is *E. amylovora*, the fire blight pathogen.

Assuming that virulent phages have been chosen from a diverse collection, using screening methods that reflect the conditions under which they are expected to perform, the ultimate test of biopesticide efficacy is the field trial, where the biopesticide is also exposed to variable environmental conditions, and the existing microbial ecology of the orchard. It is only in field trials that the efficacy and reliability of treatment can be accurately assessed, but few agricultural phage therapy studies reach this stage. Most efficacy studies are conducted on small numbers of lab-grown seedlings or young plants (Kuo et al., 1971), small cultivation chambers isolated from the applicable industrial environment (Boyd, Hildebrandt, & Allen, 1971; Munsch & Olivier, 1995), or greenhouses (Civerolo & Keil, 1969). Notable exceptions are the use of



phages to control soft-rot of calla tubers by *Pectobacterium* (formerly *Erwinia*) *carotovora* subsp. *carotovora* (Ravensdale, 2004; Ravensdale et al, 2007), bacterial spot of tomato by *Xanthomonas campestris* pv. *vesicatoria* (Balogh, 2002; Obradovic & Jones, 2004), and citrus canker caused by *Xanthomonas axonopodis* (Balogh, 2006). Ravensdale et al (2007) describe greenhouse trials, but in this case the commercial production of the crop is greenhouse-based, at least in more northern climates such as Ontario. The *Xanthomonas campestris* work is the first agricultural application of phage therapy to pass through a regulatory process and reach the commercial market, through an American company called OmniLytics (Salt Lake City, UT).

Reliability of a phage-based biopesticide is partly dependent on survival in the field environment, where viruses are subject to rapid inactivation by environmental factors (Zacchardelli et al., 1992; Schnabel & Jones, 2001; McGuire et al., 2001; Balogh, 2002; Balogh, 2006). Balogh et al (2003) significantly and substantially enhanced the long-term survival of the *Xanthomonas campestris* phages on field-grown tomato foliage by formulating the phages with skim milk and sucrose. Suspension in this colloidal medium minimized phage inactivation due to ultraviolet light and dessication. The alternative strategy described in this study uses *P. agglomerans*, a non-pathogenic epiphyte that is also susceptible to infection by *E. amylovora* phages, as a “carrier”. When both phages and carrier are applied together, the carrier should support continual phage replication, thereby limiting the amount of time that the phages are exposed to harmful environmental conditions, and increasing their total population from the time of application until the pathogen is present. The lytic activity of the phages, combined with the antagonistic effects of the *P. agglomerans* carrier, should then be able to suppress the population of and reduce the incidence of disease.

The efficacy and reliability of the biopesticide is not assured simply because of the natural setting. The variability of weather conditions greatly complicates field trials. Conditions must favour disease development at the same time that the experimental orchards are in bloom, and therefore susceptible to infection. The disease pressure, the means by which performance is evaluated, must also be reasonable. In most biopesticide field trials disease is assessed some time after a concentrated suspension of the pathogen is applied. This type of artificial inoculation is often criticized as unrealistic since the initial pathogen pressure is higher than what would generally occur in a commercial orchard. This may not be a fair test of the biopesticide, since significant effects that might be seen in a natural orchard infection may not be detected (Lindow, McGourty, & Elkins, 1996; Schnabel & Jones, 1999; Malnoy et al., 2005). However, in trials where exogenous *E. amylovora* is not applied and infection is solely dependent on natural orchard ecology, the amount of disease in the untreated controls may not be high enough to detect even a 90% reduction in disease incidence (Werner, Heidenreich, & Aldwinckle, 2004). It has been suggested that honey bees be used as a semi-natural dispersal mechanism by forcing them to traverse a passage lined with lyophilized pathogen cells as they exit the hive, but even this method produces variable levels of disease (Johnson et al., 1993b). While this may be the most realistic test, artificial inoculation produces statistically meaningful results with more consistency. Given the expense and effort of conducting field trials, the most frequently chosen compromise is to artificially inoculate trees with a moderate pathogen pressure, and to evaluate success by comparison to the standard treatment rather than to expect 100% control. Based on the pathogenicity tests in Chapters 2 and 5 of this work, the initial *E. amylovora* population in field trials should probably be less than  $1 \times 10^5$  CFU/blossom.

After all of these factors have been considered, at the root of this question of reliability is the need to understand why a particular treatment succeeds or fails at a certain time. This means monitoring the population dynamics of the biopesticide components and the pathogen over the course of field trials. In the case of the phage-carrier biopesticide for fire blight, the populations of phage, *P. agglomerans* carrier, and *E. amylovora* must be tracked. Did the biopesticide components become established in the target environment? Did they continue to flourish until the pathogen arrived? What minimum phage population was required to control the pathogen population? If the treatment fails to reduce disease, knowing the answers to these questions directs the next stage of inquiry. If the biopesticide components did not become established, the means or timing of application may need to be altered, or the conditions of cultivation may not be selecting for environmental tolerance. If they became established but were then inactivated, they may need to be applied more frequently, additives may be needed to better protect them from the environment, or the ratio of phage:carrier may need to be adjusted to optimize the dynamic predator-prey equilibrium. If the phage and carrier were well-established but still failed to control the pathogen population, there is a more fundamental problem.

TaqMan-style multiplex real-time PCR allows the populations of multiple targets to be quantified simultaneously. By using primers and probes that all have the same annealing temperature, and by using different fluorophores for each species-specific probe, we can simultaneously quantify the phage, the carrier, and the pathogen in a single reaction. This molecular method of population monitoring is faster, more specific, and more sensitive than traditional culture-based methods. To date, application of this technique to the biological control of fire blight has been limited to tracking single bacterial species, and has not been used to

explain biopesticide performance in field trials (Salm & Geider, 2004; Pujol et al., 2006).

Here, the efficacy of the phage-carrier biopesticide is tested in orchard trials and compared to the efficacy of commercially available biopesticides and to streptomycin, which most effective and most reliable means of preventing fire blight that is currently available. The population dynamics of the phages, the *P. agglomerans* carrier, and *E. amylovora* on the treated blossoms are monitored using real-time PCR, and correlated with the disease outcome of trees treated with the biopesticide.

## Methods

### *Phages and Bacterial Strains*

Refer to Chapter 2 for descriptions of all strains and isolates. Tables 7-1 through 7-3 outlines the strains used in each experiment. *E. amylovora* Ea6-4 was used to artificially infect trees in all field trials.

### *Production of Bacteria for Field Trials*

*P. agglomerans* Eh21-5 and *E. amylovora* Ea6-4 were grown and harvested as described in Chapter 2, except that 150 mm diameter Petri plates were used. Plates were flooded with 0.01 M PB before scraping cells from the surface, and the resulting suspension was decanted from the plate. Each plate produced approximately 100 mL of a  $1 \times 10^9$  CFU/mL suspension. Fresh cell suspensions were harvested each morning before use, and stored on ice.

### *Production of Phages for Field Trials*

#### a) *Growth*

Phages were grown in liquid culture on the *E. amylovora* host indicated in Table 2-1. Four-port bioreactors with a 12 L capacity (Nalgene), containing 7 L of nutrient broth were sterilized by autoclaving in pairs for 80 min at 121°C. The bioreactors were left to sit at room temperature for at least two days to ensure the sterility of the media. Just before use, bioreactors were connected to a filter-sterilized air supply as shown in Figure 7-1. The bubbled air supplied both agitation and aeration. Each bioreactor was inoculated with 140 mL of *E. amylovora* at  $1 \times 10^9$  CFU/mL in 10 mM PB. After 4 h of growth,  $1.8 \times 10^{11}$  PFU of phage

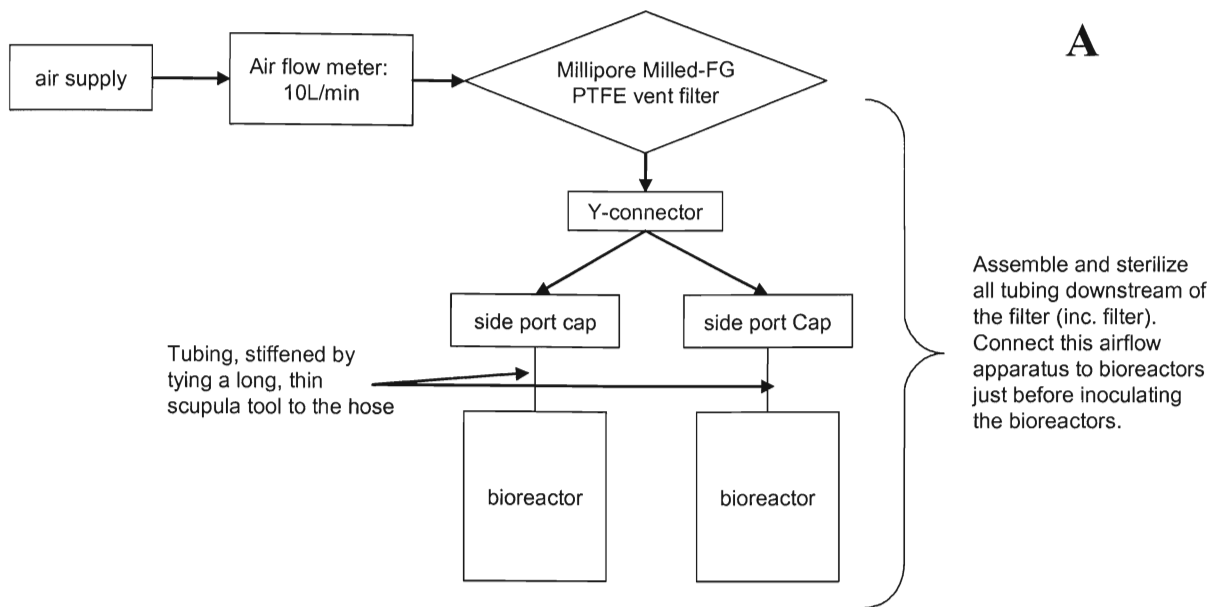


Figure 7-1. Aeration of bioreactors. A) Schematic representation of air supply setup. B) Phage cultures after 18 h growth. Note that the air flow meter and vent filter are hidden behind the first bioreactor.

were added, for a multiplicity of infection of 1. Cultures were incubated overnight, about 18 h at a room temperature of 22 to 25°C.

#### *b) Removal of Cells and Large Debris*

Bioreactors were disconnected from the air supply and processed one at a time. In general, phages were grown in pairs made up of one phage that produced large plaques with an expanding halo, and one that produced small plaques. The phage that produced large plaques was processed first to make it easier to detect cross-contamination of the two cultures.

Bacterial cells and large cell debris were mostly removed by continuous flow centrifugation at 8 000 xg, with an average outflow of about 45 mL/min. Outflow rate was controlled by clamping the outflow tubing. The bioreactor containing the crude lysate and the collection vessel were supported on ice.

To remove all remaining viable cells, the outflow was processed in batches of 2 to 30 L, by vacuum-driven filtration through a low protein-binding polyethersulfone membrane with a 0.2 µm pore size (Millipore Sterivac QP20 cartridges). The filtrate was collected in sterile media bottles with 10 mL of chloroform, and stored at 4°C for an extended period of time.

#### *c) Concentration and Buffer Replacement*

In 2005, phages were concentrated by normal flow diafiltration using an Amicon Model 8400 stirred cell apparatus (Millipore) with YM-type regenerated cellulose membrane filters. Preservatives were removed from new filters by floating them skin-side down for at least one hour, with three changes of water. Filters were used no more than 3 times. In between uses,

filters were sterilized by floating them in 70% ethanol for 20 min, then in TergAZyme for 30 min. Sterilized filters were flushed with distilled water and stored in 10% ethanol at 4°C.

The Amicon apparatus was assembled according to manufacturer's instructions, with the one gallon pressure vessel reservoir. Approximately 400 mL of distilled water was flushed through the system prior to each run. One litre each of phage lysate and distilled water were added to the reservoir. The lysate was concentrated under 50 psi of nitrogen pressure until the retentate volume was approximately 150 mL. Dissolved nutrients were partially removed by adding 600 mL of 0.01 M  $\text{PB}_{\text{salt}}$ , and again reducing retentate volume to no more than 250 mL. The ultrafiltration membrane was replaced whenever the filtrate flow rate slowed to 5 mL/min. Membranes were rinsed in a minimal volume of 0.01 M  $\text{PB}_{\text{salt}}$  to recover adsorbed phage, and the rinsate added to the ultrafiltration retentate. In order to determine the optimal membrane size, 10kDa and 100kDa membrane filters were each tested with aliquots of two phage lysates:  $\phi\text{Ea31-3}$ , a small, short-tailed phage; and  $\phi\text{Ea46-1A2}$ , a large, long-tailed phage. Flow rates were fastest across the 100 kDa membrane, with less than 0.04% loss in the filtrate. Therefore the 100 kDa membrane was used for all subsequent Amicon phage filtrations.

In 2006 and 2007, phages were concentrated by tangential flow ultrafiltration using a 30kDa cartridge, with 6 ft<sup>2</sup> regenerated cellulose membrane. The Prep/Scale-TFF apparatus (Millipore) was assembled and the cartridge was prepared according to the manufacturer's instructions. Phages were concentrated by combining 3L of the 0.2  $\mu\text{m}$  filtrate with an equal volume of sterile distilled water or 10 mM PB, reducing the total retentate volume to about 500 mL at 0.75 mbar internal pressure. Another 2 L of PB was added to the retentate, which was again reduced to about 500 mL. The system was backflushed once with PB to remove phages



adsorbed to the filtration membrane. Before processing a new phage, the cartridge was cleaned with sodium hydroxide according to the manufacturer's instructions. Total processing time for 3 L of phage suspension was about 2 hours, including filter preparation.

### *Field Trials*

Experimental orchards of *Malus X domestica* and *Pyrus communis* each consisted of 150 (Golden Delicious) or 100 (all others) trees at the Agriculture and Agri-Food Canada Research Farm in Delhi, ON. All apple cultivars were grafted on to M.9 rootstock. Pears were grafted onto seedling quince rootstock. As of 2004, Bartlett pear orchards were 4 and 12 y old respectively, Golden Delicious apple orchards were new plantings, and the east and west Fulford Gala apple orchards were 3 and 7 y old, respectively. The Idared orchard was planted in 2006, after spending 2005 in pots on Jordan Farm. Most trees were purchased from the nursery as 3 y old wood. Each Gala orchard is 0.1 ha, 4 m row spacing and 2.5 m tree spacing within each row. Each Golden Delicious orchard is 0.2 ha, with 4.5 m row spacing and 2.5 m tree spacing.

Orchards were assigned to each experiment based on the bloom stage, weather forecast, and the number of blossom clusters available. Trees were organized in a randomized complete block design, with one tree per treatment per block. Treatments were as described in Tables 7-1 through 7-3. Treatments were prepared in 1 L hand-held spray bottles, and phage-carrier treatments were left to incubate for 45 min after mixing, in order to allow phage adsorption. Application schedules were as indicated in Tables 7-1 through 7-3. Treatments were applied at noon, to near-runoff. *E. amylovora* Ea6-4 was applied to near runoff with a backpack sprayer. In 2004, a  $1 \times 10^7$  CFU/mL suspension was applied by E. Barszcz. In all other years, a  $1 \times 10^6$

CFU/mL suspension was applied by S. M. Lehman.

The incidence of diseased blossom clusters was assessed after fire blight symptoms appeared in the controls. Incidence was assessed on a per cluster basis. If necrosis extended to the base of the peduncle in at least one blossom in a given cluster, that cluster was considered diseased. If all blossoms had fallen off, the remaining stump and surrounding tissue at the cluster base was examined for necrosis. The percent of treated clusters that were diseased was recorded.

Temperature and relative humidity within the canopy were monitored using HOBO environmental monitors (Onset Computer Corporation) positioned throughout the orchard, protected by weather shields. Data were recorded every 15 min. Data from different monitors were pooled. There was little variation between distantly spaced orchards (ie. Idared and Gala) therefore data from a given time period was considered valid for all orchards in Delhi.

Two trials were conducted in 2004, one in 4 y old Bartlett pear and one in newly planted Golden Delicious (Table 7-1). In the pear trial there were 7 blocks, with 10 treatments per block. In the apple trial there were 8 blocks, each with 12 treatments. BlightBan A506, a commercially available *P. fluorescens*-based bacterial antagonist, was provided by Nufarm Agricultural Products (Calgary, AL).

Three field trials were conducted in 2005 (Table 7-2). Fresh samples of BlightBan C9-1, a commercially available *P. agglomerans*-based bacterial antagonist, and BlightBan A506 were provided by Plant Health Technologies (Boise, ID). Green Julius Plant Wash and instructions for its use were provided by Lorne Allin, of LifeTime Solutions (Newcastle, ON).

Table 7-1. Experimental design of 2004 field trials.

Treatment Type <sup>a</sup>	Orchard	
	Bartlett Pear (4 y old)	Golden Delicious (new)
Controls	Tap water; 100 ppm a.i. Streptomycin 17	Tap water; 100 ppm a.i. Streptomycin 17
Bacterial Antagonists <sup>b</sup>	BlightBan A506; <i>P. agglomerans</i> 21-5	BlightBan A506; <i>P. agglomerans</i> 21-5
Phage-Carrier <sup>b</sup>	Eh21-5 + $\phi$ Ea9-5; Eh21-5 + $\phi$ Ea21-4; Eh21-5 + $\phi$ Ea35-4; Eh21-5 + $\phi$ Ea46-1A2; Eh21-5 + $\phi$ Ea9-5 (1/20th volume); Eh21-5 + $\phi$ Ea46-1A2 (1/20th vol.)	Eh21-5 + $3 \times 10^2$ PFU/mL $\phi$ Ea9-5; Eh21-5 + $3 \times 10^2$ PFU/mL $\phi$ Ea21-4; Eh21-5 + $3 \times 10^2$ PFU/mL $\phi$ Ea45-1B; Eh21-5 + $3 \times 10^2$ PFU/mL $\phi$ Ea46-1A2; Eh21-5 + $5 \times 10^5$ PFU/mL $\phi$ Ea51-2; Eh21-5 + $5 \times 10^5$ PFU/mL $\phi$ Ea31-3; Eh21-5 + $5 \times 10^7$ PFU/mL $\phi$ Ea35-4; Eh21-5 + $5 \times 10^6$ PFU/mL $\phi$ Ea35-4;
Treatment Application	80-100% bloom (11 May)	50-70% bloom (19 May)
Pathogen Application	100% bloom (12 May)	80-100% bloom (20 May)

<sup>a</sup> All treatments were prepared with tap water.

<sup>b</sup> All bacterial suspensions were prepared to  $1 \times 10^8$  CFU/mL. The concentrations of the phage suspensions used in the pear orchard were unknown.

Table 7-2. Experimental design of 2005 field trials.

Treatment Type <sup>a</sup>	Orchard		
	Bartlett Pear (12 y)	Fulford Gala (4 y old)	Fulford Gala (8 y old)
Controls	10 mM PB; 100 ppm Streptomycin	10 mM PB; 100 ppm Streptomycin	10 mM PB; 100 ppm Streptomycin
Bacterial Antagonists <sup>b</sup>	BlightBan A506; BlightBan C9-1; <i>P. agglomerans</i> Eh21-5	BlightBan A506; BlightBan C9-1; <i>P. agglomerans</i> Eh21-5	<i>P. agglomerans</i> Eh21-5
Phage-Carrier <sup>b</sup>	Eh21-5 + $\phi$ Ea21-4; Eh21-5 + $\phi$ Ea45-1B; Eh21-5 + $\phi$ Ea46-1A2; Eh21-5 + $\phi$ Ea45-1B + $\phi$ Ea46-1A2	Eh21-5 + $\phi$ Ea21-4; Eh21-5 + $\phi$ Ea45-1B; Eh21-5 + $\phi$ Ea46-1A2 Eh21-5 + $\phi$ Ea45-1B + $\phi$ Ea46-1A2	10 <sup>6</sup> Eh21-5 + $\phi$ Ea46-1A2 (MOI=0.1); 10 <sup>6</sup> Eh21-5 + $\phi$ Ea46-1A2 (MOI=1); 10 <sup>6</sup> Eh21-5 + $\phi$ Ea46-1A2 (MOI=10); 10 <sup>8</sup> Eh21-5 + $\phi$ Ea46-1A2 (MOI=0.1); 10 <sup>8</sup> Eh21-5 + $\phi$ Ea46-1A2 (MOI=1)
Other			Green Julius Plant Wash (1:100 dilution)*
Treatment Application	90-100% bloom (19 May)	30-40% bloom (20 May) 40-50% bloom (21 May)	30-40% bloom (20 May) 40-50% bloom (21 May)
Pathogen Application	100% bloom (21 May)	50-70% bloom (22 May)	50-70% bloom (22 May)

<sup>a</sup> All treatments were prepared in PB except for streptomycin, Green Julius, and BlightBan, which were prepared in tap water according to the manufacturer's instructions.

<sup>b</sup> Bacteria and phage were prepared to final concentrations of  $1 \times 10^8$  CFU/mL, unless otherwise indicated.

Table 7-3. Experimental design of 2006 field trials.

Treatment Type <sup>a</sup>	Orchard		
	Fulford Gala (5 y old)	Fulford Gala (9 y old)	Idared (new)
Controls	10 mM PB; 100 ppm Streptomycin	10 mM PB; 100 ppm Streptomycin	10 mM PB
Bacterial Antagonists <sup>b</sup>	BlightBan C9-1; BlightBan A506; <i>P. agglomerans</i> Eh21-5	BlightBan C9-1; <i>P. agglomerans</i> Eh21-5	BlightBan C9-1; <i>P. agglomerans</i> Eh21-5
Phage-Carrier <sup>b</sup>	Eh21-5 + $\phi$ Ea21-4; Eh21-5 + $\phi$ Ea45-1B; Eh21-5 + $\phi$ Ea46-1A2; Eh21-5 + $\phi$ Ea45-1B + $\phi$ Ea46-1A2	Eh21-5 + $\phi$ Ea10-1; Eh21-5 + $\phi$ Ea10-6; Eh21-5 + $\phi$ Ea31-3; Eh21-5 + $\phi$ Ea35-4; Eh21-5 + $\phi$ Ea31-3 with Physpe overspray	Eh21-5 + $\phi$ Ea45-1B; Eh21-5 + $\phi$ Ea46-1A2
Treatment Application <sup>c</sup>	30-40% bloom (13 May) 40-50% bloom (14 May)	30-40% bloom (13 May) 40-50% bloom (14 May)	80-100% bloom* (10 May)
Pathogen Application <sup>c</sup>	50-70% bloom (15 May)	50-70% bloom (15 May)	NA
Treatment Type <sup>a</sup>	Orchard		
	Golden Delicious (new - north)	Golden Delicious (new - south)	
Controls	10 mM PB; 100 ppm Streptomycin	10 mM PB; 100 ppm Streptomycin	
Bacterial Antagonists <sup>b</sup>	<i>P. agglomerans</i> Eh21-5	<i>P. agglomerans</i> Eh21-5	
Phage-Carrier <sup>b</sup>	Eh21-5 + $\phi$ Ea46-1A2 + $\phi$ Ea21-4 + $\phi$ Ea31-3; Eh21-5 + $\phi$ Ea21-4 + $\phi$ Ea31-3 + $\phi$ Ea35-4; Eh21-5 + $\phi$ Ea31-3 + $\phi$ Ea35-4 + $\phi$ Ea10-1; Eh21-5 + $\phi$ Ea35-4 + $\phi$ Ea10-1 + $\phi$ Ea46-1A2;	Eh21-5 + $\phi$ Ea21-4 (30 min); Eh21-5 + $\phi$ Ea21-4 (3:30 min); Eh21-5 + $\phi$ Ea31-3 (30 min); Eh21-5 + $\phi$ Ea31-3 (3:30 min); Eh21-5 + $\phi$ Ea46-1A2 (30 min); Eh21-5 + $\phi$ Ea46-1A2 (3:30 min)	
Other	Inventek soap (1:124 v/v); kasumin (84 ppm)		
Treatment Application <sup>c</sup>	30-40% bloom (13 May) 40-50% bloom (14 May)	30-40% bloom (13 May) 40-50% bloom (14 May)	
Pathogen Application <sup>c</sup>	50-70% bloom (15 May)	50-70% bloom (15 May)	

<sup>a</sup> All treatments were prepared in PB (pH 6.8) except for streptomycin, Green Julius, and BlightBan, which were prepared in tap water according to the manufacturer's instructions.

<sup>b</sup> Bacterial and phage were prepared to final concentrations of  $1 \times 10^8$  CFU or PFU/mL, unless otherwise indicated. If more than one phage was used, the summed concentration of virions was  $1 \times 10^8$  PFU/mL.

<sup>c</sup> Individual clusters were flagged for treatment and followup assessment if most blossoms in the cluster were fully open. Therefore, this note reflects the bloom stage of the treated clusters, not the orchard as a whole.

Five field trials were conducted in 2006 (Table 7-3). Sufficient amounts of the BlightBan products remained in storage from 2005. Their viability was tested by suspending 1 g of each formulation in 10 mL of PB for 15 min, and then plating serial dilutions of each suspension on nutrient agar. The culturable concentration of BlightBan C9-1 was unchanged from the time of packaging. The culturable concentration of BlightBan A506 was 10-fold lower than the  $1 \times 10^{11}$  CFU/g stated on the label. The amount of BlightBan A506 used was adjusted accordingly, in order to achieve a final concentration of  $1 \times 10^8$  CFU/mL. A new formulation of bactericidal soap, called Inventek, and instructions for its use were provided by Lorne Allin, of LifeTime Solutions Inc. Physpe is an SAR inducer produced by Göemar Laboratories (Saint-Malo, FR).

### *Population Monitoring*

During 2004, blossom populations of *P. agglomerans* were estimated by plate counts. Three blossoms from different locations on each pear tree in block 2. Each set of three blossoms was sonicated for 2 min in 10 mL of 10 mM PB. The blossom wash was serially diluted in PB and 100  $\mu$ L were plated on both nutrient agar and modified Miller-Schroth medium (Brulez & Zeller, 1981). Total *P. agglomerans* populations were estimated based on the number of yellow bacterial colonies on nutrient agar, which were the dominant colony type. These counts were similar to the total number of orange, EPS-producing colonies on MMS. MMS is a semi-selective media, on which *P. agglomerans* and *Erwinia amylovora* produce characteristic colony types. Blossoms were sampled 20 h after treatment application, and 4 d after treatment application.

Very few blossoms were open in the Golden Delicious orchard, therefore one blossom

was sampled from each tree in blocks 1, 3, 5, and 7, 20 h after treatment application. Bacterial populations were collected in 1 mL PB. Total *P. agglomerans* populations were estimated based on the number of yellow colonies on nutrient agar. The identity of these colonies was confirmed by replica plating on CCT, a semi-selective medium on which *P. agglomerans* has a characteristic appearance. Pre-treatment populations in both orchards were estimated from 8 trees distributed evenly through the orchard.

During 2005 and 2006, multiplex real-time PCR was used to monitor the populations of *P. agglomerans*, bacteriophages, and *E. amylovora* on the blossom surfaces at three time points during the experiment: before BCA application, immediately following first BCA application, and immediately before application of the pathogen. In 2005, three blossoms were sampled from each tree, in each of three blocks. The petals were removed from each blossom, the remaining blossom heads were plucked from the petiole and placed in a sterile, capped plastic culture tube. Samples were transported on ice, and stored overnight at 4°C before analysis.

Direct Plant Extraction Buffer (DiPEB, Cat. No. 00690, Agdia Inc., Elkhart, IN) was added to each sample in a ratio of 1 mL buffer per blossom. Samples were sonicated for 2 min, and a 1 mL aliquot of each was centrifuged at 10 000 xg for 10 min at 4°C. The resulting pellet was resuspended in 100 µL of wash buffer and used 3 µL as the template for real-time PCR reactions.

### *Real-time PCR*

Primers and TaqMan-style probes were designed by Dr. W. -S. Kim (unpublished data) and were as described in Table 6-1, except that the  $\phi$ -dpo2 probe and primers described in Chapter 2 were used for phage detection.

All reactions were conducted in a 25  $\mu$ L total volume. In 2005, reactions were carried out under the following conditions: 95°C for 10 min for initial denaturation, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s, with 3 endpoint fluorescence readings taken during the amplification step. Each reaction contained 1X Brilliant QPCR Multiplex Master Mix (Stratagene), 200  $\mu$ M each primer, 100  $\mu$ M each probe, 3  $\mu$ L template.

In 2006, reactions were carried out under the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 16 s, with 2 endpoint fluorescence readings taken during the amplification step. Each reaction contained 200 nM each primer, 100 nM each probe, 3  $\mu$ L template.

Standard curves were constructed using serially diluting cell or phage suspensions in clean blossom wash, either individually or as mixtures. Standard curve construction was repeated using new cultures. PCR reactions were run in triplicate.

### *Statistical Analysis*

Regression analysis of standard curve data was conducted in SigmaPlot, version 8 (SPSS, Inc., Chicago, IL). All other data were analyzed using SAS (Statistical Analysis Systems 8.2; SAS Institute, Cary, NC). Disease incidence was analyzed using the general linear model (PROC GLM). A one-sided Dunnett-Hsu test ( $\alpha = 0.01$ ) was used to evaluate the reduction in disease



relative to the water or buffer control. Differences between treatments were assessed using a two-sided Tukey-Kramer test ( $\alpha = 0.05$ ). Very little disease was observed in the 2004 Golden Delicious trial, and it developed too late in the season to directly relate symptoms to specific blossom clusters. Therefore the presence or absence of disease was modeled using PROC GENMOD, with a binomial distribution. To compensate for overdispersion, the scale parameter was estimated from the square root of the ratio of Pearson's  $\chi^2$  statistic to degrees of freedom.

## Results

### *Sensitivity and Specificity of Real-time PCR*

The specificity of the multiplex real-time PCR primers and probes is shown in Table 7-4. The *lsc* gene is present on the chromosome of *E. amylovora*, therefore all strains were detected regardless of whether they carry the pEA29 plasmid.

Multiplex standard curves for *E. amylovora* and *E. pyrifoliae* are shown in Figure 7-2. Concentrations higher than  $3 \times 10^6$  CFU per 25  $\mu$ L reaction were not tested. The relationships between threshold cycle ( $C_T$ ) and initial quantity were linear across a 4-log range.

The standard curves for both species are essentially identical; the slight differences between slope and intercept values were within the range of variation between replicate real-time PCR runs. As in Chapter 4, there is no significant difference between the standard curves produced in singleplex vs. multiplex reactions.

Standard curves were prepared from cells diluted in buffer that had been sonicated with uninoculated blossoms according to the sampling procedure used for the field trials. The curves therefore correspond directly to initial cell numbers, and account for the presence of any PCR inhibitors that may be present in the experimental samples. Since blossom infection occurs through the stigma and hypanthium, petals and peduncles were removed from each blossom, and bacteria were collected from the surfaces of the remaining structures. Removal of petals and peduncles improved the sensitivity of bacterial quantification (data not shown), presumably because it reduced the total volume of plant tissue, and thus the concentration of plant-derived PCR inhibitors. DNA extraction was not performed as part of sample preparation. The components of the wash buffer, along with the 5 min initial denaturation cycle at 95°C, were

Table7- 4. Specificity of the real-time PCR primers and probes used in this study. All strains were tested in a multiplex reaction.

Species Tested	Strain	Host Plant	Origin	Signal from Primers and Probe designed for:			Original Strain Reference or Source	
				<i>E. amylovora</i>	<i>P. agglomerans</i>	Phage		
<i>Erwinia amylovora</i>	with pEA29 plasmid	Ea6-4	<i>Pyrus sp.</i>	Canada	+	-	-	AAFC-Vineland
		Ea110	<i>Malus sp.</i>	Michigan, USA	+	-	-	Ritchie and Klos, 1977
		Ea273	<i>Malus sp.</i>	New York, USA	+	-	-	Beer (1971)
		Ea1/79	<i>Cotoneaster sp.</i>	Germany	+	-	-	Falkenstein <i>et al.</i> , 1988
		1H3-1	<i>Raphiolepis indica</i>	Louisiana, USA	+	-	-	Holcomb, 1998
	without pEA29 plasmid	LebB66	<i>Malus sp.</i>	Lebanon	+	-	-	Saad, 2000
		UTRJ2	<i>Malus sp.</i>	Utah, USA	+	-	-	Thomson and Ockey, 2001
		1614-2a	<i>Crataegus sp.</i>	Spain	+	-	-	Llop et al, 2004
<i>Pantoea agglomerans</i>	Eh21-5	<i>Pyrus sp.</i>	Canada	-	+	-	this work	
	Eh1-28b	<i>Malus sp.</i>	Canada	-	+	-	AAFC-Vineland	
	33243	not specified	Canada	-	+	-	American Type Culture Collection	
	49018	<i>Malus sp.</i>	not specified	-	+	-	American Type Culture Collection	
	2696	<i>Prunus armeniaca</i>	Michigan, USA	-	+	-	International Collection of Microorganisms from Plants	
	C9-1	<i>Malus sp.</i>	Michigan, USA	-	+	-	Ishimaru <i>et al.</i> , 1988	
	E325	<i>Malus sp.</i>	Washington, USA	-	+	-	Pusey, 1997	
	Bacteriophages of <i>E. amylovora</i>	NEa1	<i>Malus sp.</i>	Michigan, USA	-	-	+	Ritchie, 1978
NEa31-3		soil	Canada	-	-	+	Gill, 2000	
NEa46-1		soil	Canada	-	-	+	Gill, 2000	
NEa45-1		soil	Canada	-	-	+	Gill, 2000	
<i>Erwinia pyrifoliae</i>	Ep1/96	<i>Pyrus pyrifoliae</i>	Korea	-	-	-	Kim et al., 1999	
<i>Erwinia carotovora</i>	Ecc26			-	-	-	D. Cupples	
<i>Escherichia coli</i>	DH-5"			-	-	-	A. J. Castle	
<i>P. fluorescens</i>	A506			-	-	-	Lindemann and Suslow, 1987	

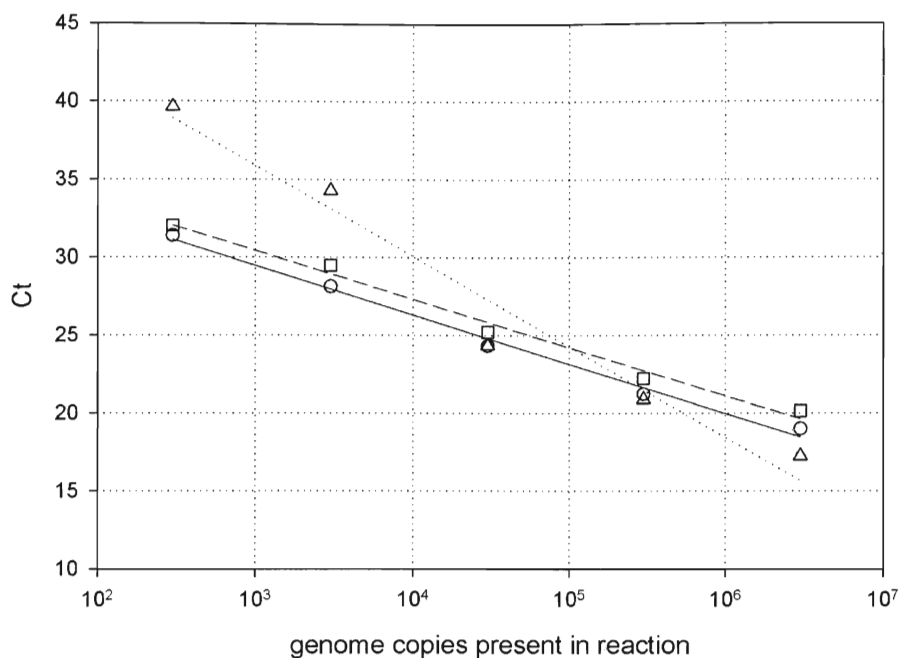


Figure 7-2. Standard curves for the detection of *E. amylovora* (open boxes, solid line), *P. agglomerans* (open circles, dashed line), and phages (open triangles, dotted line) in a multiplex reaction. Quantification is given by the equations  $y = 47.57 - 3.11 \log(x)$ ;  $R^2 = 0.987$  (*E. amylovora*),  $y = 46.98 - 3.17 \log(x)$ ;  $R^2 = 0.992$  (*P. agglomerans*), and  $y = 68.01 - 5.82 \log(x)$ ,  $R^2 = 0.962$  (phage).

sufficient to make the bacterial target DNA accessible for amplification.

### *Production of Phages for Field Trials*

The concentration of two phages was monitored through each stage of production in 2005.  $\phi$ Ea46-1A2 is a long-tailed phage with a large head, and  $\phi$ Ea31-3 is a short-tailed phage with a small head. The concentrations of these phages in two independent trials, conducted with different initial concentrations of phage, are given in Table 7-5.

Very slight losses were observed across the 0.2  $\mu$ m membrane. In trial 1, the net volume of the  $\phi$ Ea31-3 0.2  $\mu$ m filtrate was reduced 10-fold across the 100 kDa membrane. In all other cases the net volume of the 0.2  $\mu$ m filtrate was reduced 5-fold across the 100 kDa membrane. The relative concentrations of the two fractions are generally consistent with their relative volumes. No net changes in retentate volume was made using the 50 kDa membrane. In trial 1, there was less than a 0.04% loss of total viable phages to the 100 kDa filtrate, even for the smaller phage. Larger losses were observed across the 100 kDa membrane in trial 2, 7% for  $\phi$ Ea46-1A2 and 0.2% for  $\phi$ Ea31-3. Additional losses of phage, apparently by adsorption to the membrane or physical damage, were observed during certain diafiltration steps. There was a substantial loss of  $\phi$ Ea31-3 during the 50kDa filtration in trial 1, and a 25% loss of  $\phi$ Ea31-3 to the 100 kDa membrane.

In 2006, tangential flow filtration was used to concentrate the phages instead of normal flow diafiltration. With a 100 kDa membrane, up to 10% of  $\phi$ Ea31-3 was lost to the filtrate (data not shown). Switching to a 30kDa membrane reduced this loss to less than 0.1 %.

Table 7-5. Concentration and loss of phages throughout production and purification.

Phage	Fraction	Concentration (PFU/mL)	
		Trial 1	Trial 2
$\phi$ Ea46-1A2	After continuous flow centrifugation	NT <sup>a</sup>	$2.0 \times 10^9$
	0.2 $\mu$ m Millipore filtrate	$8 \times 10^7$	$1.7 \times 10^9$
	100 kDa diafiltration retentate	$6 \times 10^8$	$9.0 \times 10^9$
	100 kDa diafiltration filtrate	$1.2 \times 10^4$	$6.5 \times 10^7$
	50 kDa diafiltration retentate	NT	$6.0 \times 10^9$
	50 kDa diafiltration filtrate	NT	$1.8 \times 10^7$
$\phi$ Ea31-3	After continuous flow centrifugation	NT	$7.3 \times 10^9$
	0.2 $\mu$ m Millipore filtrate	$6 \times 10^3$	$6.6 \times 10^9$
	100 kDa diafiltration retentate	$6.7 \times 10^4$	$2.5 \times 10^{10}$
	100 kDa diafiltration filtrate	0	$6.5 \times 10^6$
	50 kDa diafiltration retentate	$2.0 \times 10^1$	$2.5 \times 10^{10}$
	50 kDa diafiltration filtrate	0	$3.8 \times 10^6$

<sup>a</sup> “NT” indicates that the fraction was not tested.

*Disease Incidence and Microbial Population Dynamics - 2004*

Disease incidence in the pear orchard is shown in Figure 7-3; the results were highly variable among different blocks. Disease incidence in the control treatment was 31.4% of clusters. None of the treatments were significantly different from the water control ( $P > 0.3$ , Tukey-Kramer). Disease symptoms progressed rapidly through these trees, and the orchard had to be removed. Very little disease developed in the Golden Delicious orchard, even in the buffer control, and very few blossom clusters were present in these young trees. Therefore disease was recorded as presence or absence in the tree as a whole. There was no significant difference between the likelihood of disease in any of the treatments and likelihood of disease in the water control ( $P > 0.09$ , PROC GENMOD).

Despite the lack of treatment efficacy in 2004, there does appear to have been a short-term effect of some treatments on the blossom populations of *P. agglomerans* (Figure 7-4A). Twenty hours after treatments were applied to the pear orchard, the mean *P. agglomerans* populations on trees treated with streptomycin, BlightBan A506, or carrier with  $\phi$ Ea21-4 were lower than the water-treated trees by a factor of  $10^2$  to  $10^3$ , and were at least 10-fold lower than the pre-treatment populations the day before. Twenty hours after treatments were applied to the apple orchard, *P. agglomerans* was not detected on BlightBan A506-treated trees (Figure 7-43B). The mean *P. agglomerans* populations on trees treated with streptomycin or carrier with  $\phi$ Ea45-1B were at least  $10^2$ -fold lower than any other treatments, including the control, but still within an order of magnitude of the pre-treatment populations.

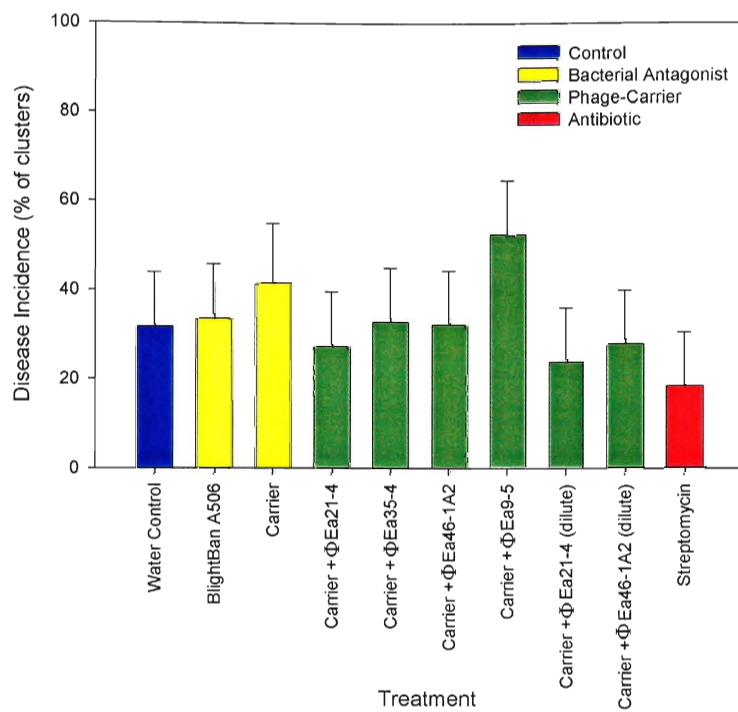


Figure 7-3. Incidence of fire blight in 4 y old Bartlett pear (2004).



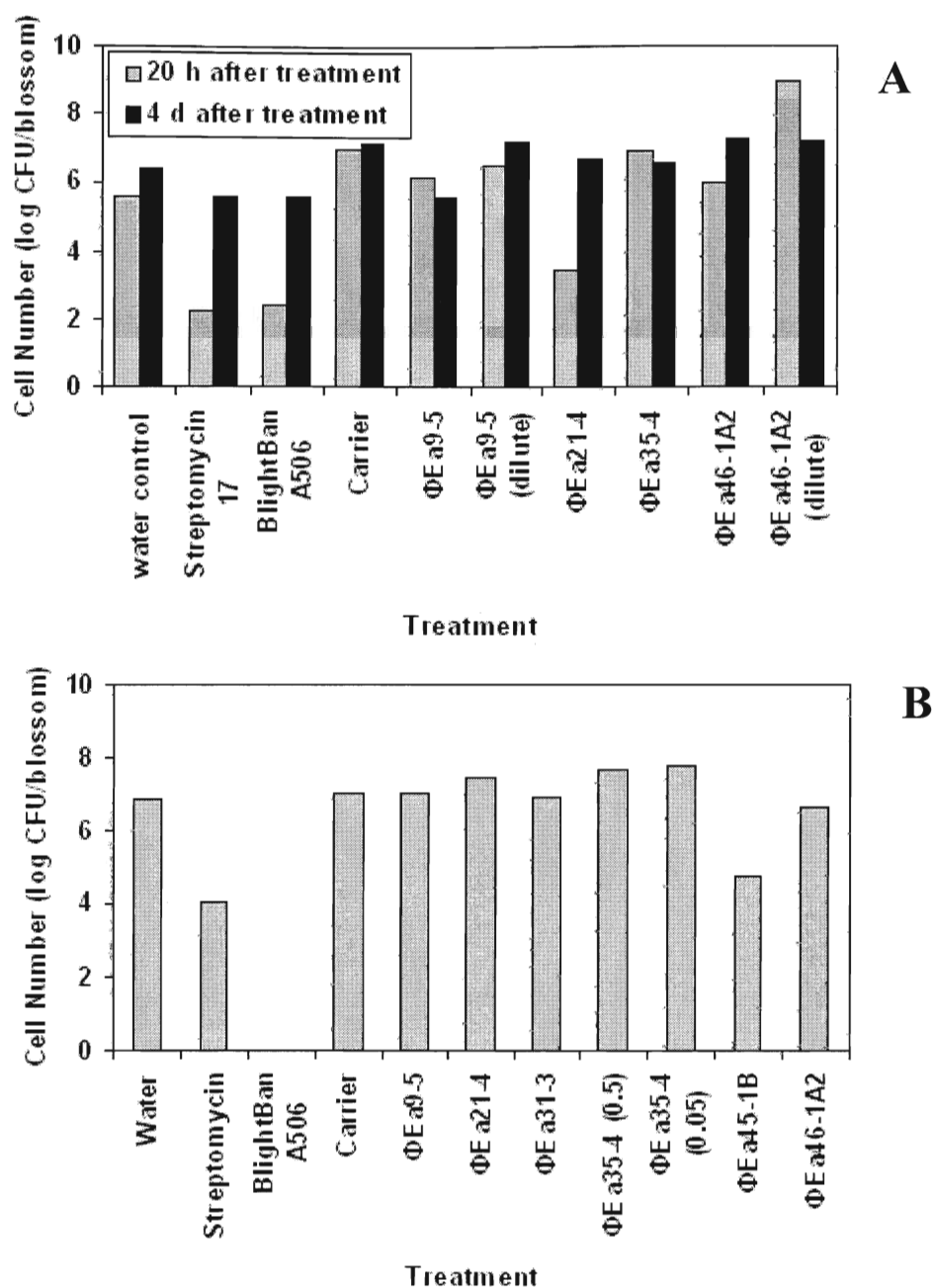


Figure 7-4. *P. agglomerans* populations on A) 4 y old pear, and B) new Golden Delicious apple blossom hypanthia during 2004 field trials. In panel A, the initial MOI was 1 for all phage-carrier treatments except those marked “(dilute)”. The MOI for treatments in panel B was variable (see Table 7-1), and is only stated here to distinguish between the different applications of  $\phi$ Ea35-4. The mean population immediately before treatment was  $1.5 \times 10^4$  CFU/blossom in the pear orchard, and  $8.6 \times 10^4$  CFU/blossom. All phages were mixed with the carrier prior to application.

*Disease Incidence and Microbial Population Dynamics - 2005*

In 2005, three field experiments were conducted in apple and pear orchards (100 trees/orchard, randomized complete block design). Disease incidence in the buffer-treated controls was approximately 50% in the two apple trials, and 72% in the pear trial (Figure 7-5 and 7-6). Streptomycin, which is considered the gold standard for fire blight prevention, significantly reduced the incidence of disease in all three experiments.

The efficacy of BlightBan products was variable. In the 12 y old pears that were treated once, neither BlightBan A506 nor BlightBan C9-1 caused a significant reduction in disease incidence (Figure 7-5A). In the apple orchard, only BlightBan C9-1 was effective (Figure 7-5B).

The Green Julius bactericidal soap was highly effective (Figure 7-6). Disease incidence in Green Julius-treated apple trees was 27.2%, which was significantly less than in the control trees ( $p=0.0048$ , Dunnett-Hsu test).

Three phages were applied with the *P. agglomerans* Eh21-5 carrier, in various combinations. Across all experiments, 6 out of 12 phage-based treatments significantly reduced disease incidence relative to the water-treated control ( $P < 0.01$ ). In the 12 y old pear and east Gala orchards, the carrier alone did not cause a significant reduction in disease incidence, while certain phage-carrier combinations did (Figure 7-5). However, different phage-carrier combinations were effective in each orchard. In the west Gala orchard, the effects of the total and relative populations of phage and carrier were tested. Only two phage-carrier treatments in this trial were effective, the treatment consisting of  $1 \times 10^6$  CFU/mL carrier with  $1 \times 10^5$  PFU/mL phage, and the treatment consisting of  $1 \times 10^8$  CFU/mL carrier with  $1 \times 10^8$  PFU/mL of phage (Figure 7-6).

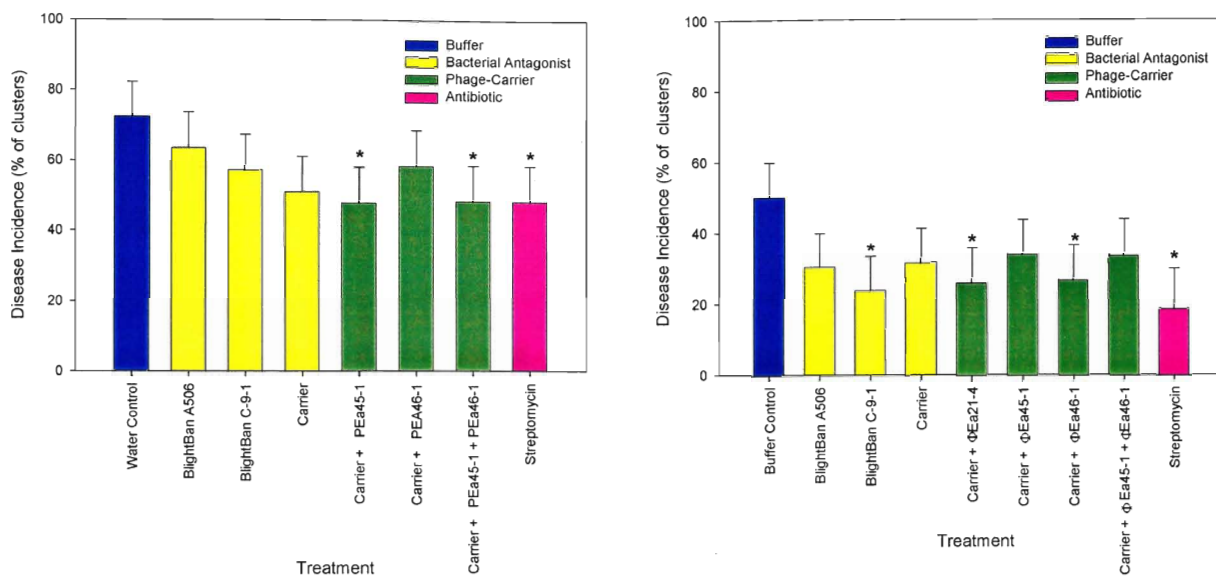


Figure 7-5. Incidence of fire blight in A) 12 y old Bartlett pear and B) 4 y old Gala apple orchards (2005). Asterisks indicate significantly less disease than in the control.

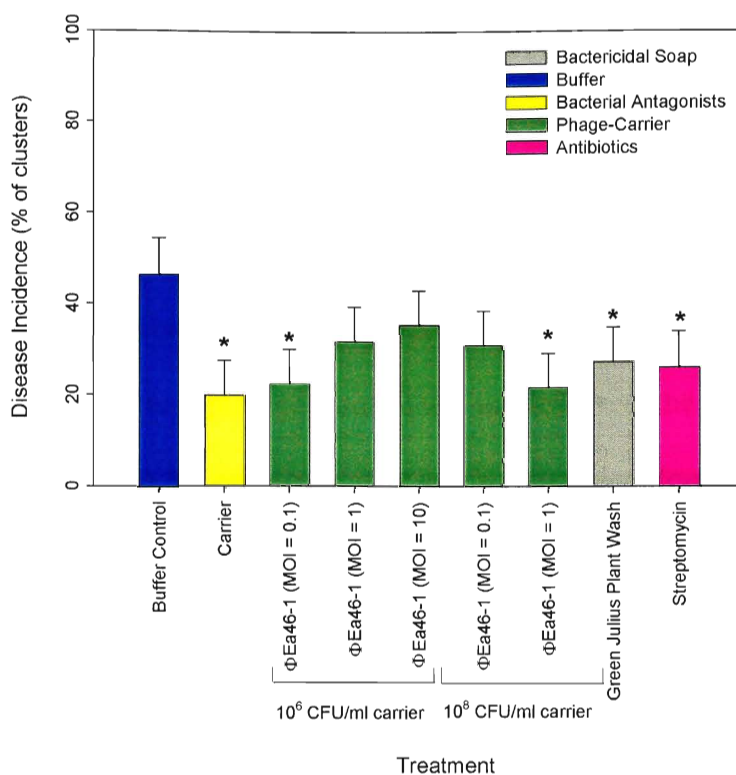


Figure 7-6. Incidence of fire blight in 8 y old Gala apple treated with varying amounts of  $\phi$ Ea46-1A2 in different carrier:phage ratios (2005). Asterisks indicate significantly less disease than in the control.

In the 2005 field trials, multiplex Real-time PCR was used to monitor the populations of the carrier, two phages, and the pathogen in two experimental orchards (Figure 7-7). Samples were collected immediately before and after initial treatment application, immediately after pathogen application, and 3 days after pathogen application. In all cases, disease outcome was correlated with an *E. amylovora* population of less than  $1 \times 10^4$  CFU/blossom 3 days after its application. In all cases where significant control was not achieved, the total *P. agglomerans* population was lower than in the one experiment where the carrier-alone treatment was successful; and/or if phage had been applied, the phage population showed a net decline by the time pathogen was applied. In all cases where significant control was achieved, if phage had been applied, there had been a net increase in the phage population by the time pathogen was applied (at the expense of the *P. agglomerans* population). The phage population then continued to increase, but now growing preferentially on the pathogen. If phage had not been applied, the *P. agglomerans* population was larger than in unsuccessful treatments.

In all experiments, the efficacy of successful treatments was not statistically different from the efficacy of streptomycin ( $P < 0.05$ , Tukey-Kramer test), which is the most effective bactericide currently available. This amounted to a 50% reduction in disease incidence in apple trees, and a 33% reduction in pear trees.

#### *Disease Incidence and Microbial Population Dynamics - 2006*

A severe, unforecasted frost four days after treatment resulted in the destruction of all open blossoms in the experimental orchards. As a result, no data were collected, and these experiments are scheduled to be repeated in 2007. However, within one day of treatment,

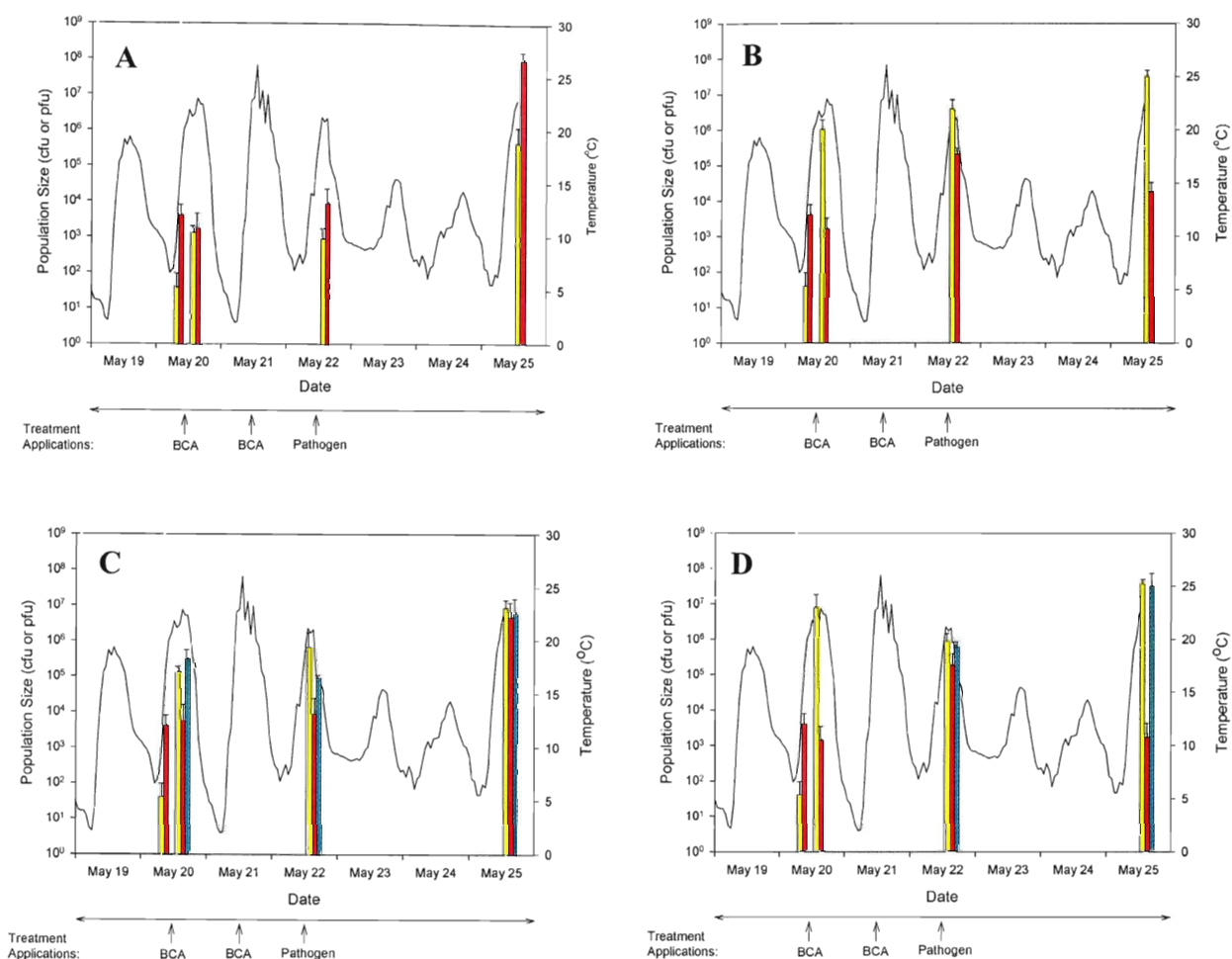


Figure 7-7. Population dynamics of *E. amylovora* (red bars), *P. agglomerans* (yellow bars), and *Erwinia* phage  $\phi$ Ea46-1A2 (blue bars) on 8 y old Gala blossoms (2005). Population sizes are the average numbers of each species present on the stigma, anthers, and hypanthium of each blossom, based on composite samples from each of 3 experimental blocks. Timing of biocontrol agent (BCA) and pathogen applications are indicated below the x-axis. Temperature (solid black line) was recorded throughout all experiments. (A) Buffer-treated trees; (B) Trees treated with the *P. agglomerans* carrier alone; (C) Trees treated with a mixture of  $1 \times 10^6$  CFU/mL *P. agglomerans* and  $1 \times 10^5$  PFU/mL  $\phi$ Ea46-1A2; (D) Trees treated with a mixture of  $1 \times 10^8$  CFU/mL *P. agglomerans* and  $1 \times 10^8$  PFU/mL  $\phi$ Ea46-1A2.

moderate to severe phytotoxicity was observed on blossoms treated with the bactericidal soap.

### *Weather Conditions*

Figure 7-8 shows the weather conditions that were monitored in the experimental orchards during the 2004 and 2005 field trials. High relative humidities were recorded throughout all experiments, and were generally associated with nightly dew. However, the recorded temperatures varied substantially among these three experiments.

The daytime temperatures in early May of 2004 (panel A) were well within the optimum growth range for *E. amylovora*. The experimental orchard, 100 trees of 4 y old Bartlett pear, was so severely diseased that it was destroyed. These same conditions also led to the eruption of shoot blight in an adjacent orchard of 11 y old Bartlett pear trees that had been infected with *E. amylovora* in previous years, but which had been asymptomatic at the time. Thirty percent of that orchard was destroyed, and the remaining trees were heavily pruned in order to remove diseased tissue.

Later in the same month, a similar experiment was conducted in the 1 y old Golden Delicious orchard. The average daily temperatures were lower than they were during the pear experiment, but were still well within the optimum growth range for *E. amylovora* on most days. In this case, however, the temperature dropped sharply, to less than 5°C, within 7 h of pathogen application. For the rest of the season, only one or two infected blossom clusters were observed among the 150 trees in this orchard, but evidence of latent infection was observed in 50% of the trees at the start of the following spring.

The average daily temperatures in 2005 (panel B) appear marginal when considering only

the optimum pathogen growth range, but were really quite moderate when the total growth range is considered, particularly on the day of pathogen application and the day immediately after it. A 43% incidence of disease was observed in the control trees in this orchard (Figure 7-4). Moderate pruning was required to remove the diseased tissue from these 3 to 7 y old Gala trees, and none had to be destroyed.

The temperatures during the 2006 trials (panel D) were similar to those in 2005 (panel B), but a frost on May 23 killed all of the open blossoms. The dead blossoms fell off of the trees and no disease data could be collected.



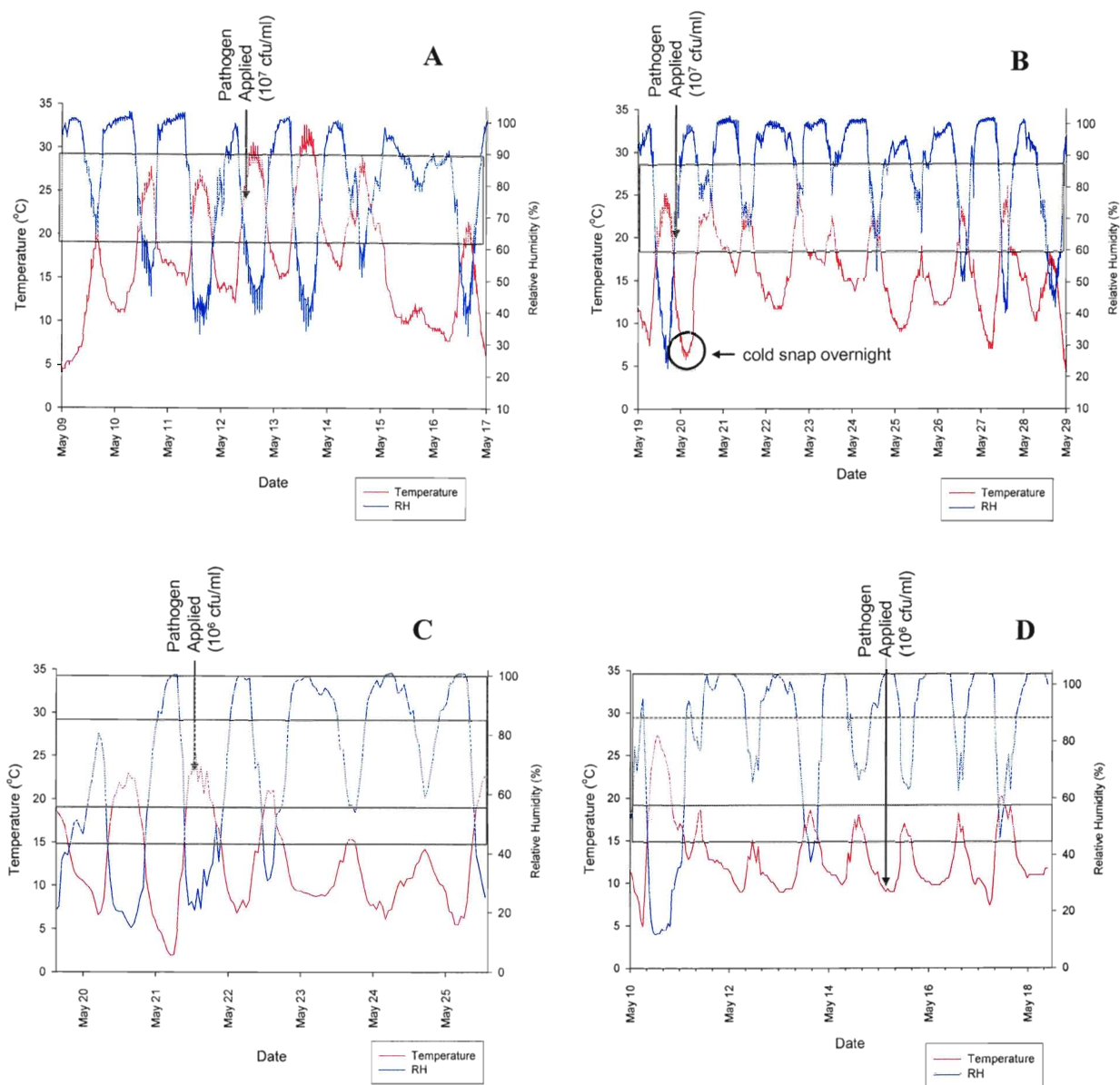


Figure 7-8. Orchard weather conditions during the A) 2004 4 y old pear trial, B) 2004 Golden Delicious apple trial, C) 2005 Gala trial, and D) 2006 trials. The date of pathogen application, and the concentration of the *E. amylovora* suspension that was applied are indicated. Note that a higher pathogen pressure was applied during 2004. The optimum growth temperature range of *E. amylovora* is indicated for all years. The broader growth range of *E. amylovora* is shown in panels B and D.

## Discussion

The phage production methods developed during the course of these field trials demonstrate that larger-scale batch culture and processing is possible. Phage concentrations in the partially clarified crude lysate (following continuous flow centrifugation) were on the order of  $1 \times 10^9$  PFU/mL. Ideally, this should be about 10-fold higher, but it was sufficient for the needs of these field trials, and is about the average for many types of phages cultured in volumes of 0.5 to 10 L (Munsch & Olivier, 1995; Civerolo & Keil, 1969; Civerolo, 1970). The greater loss of phages to filtrate using a tangential vs. normal (ie. Amicon) flow system with the same membrane pore size suggests that phage retention by the 100 kDa membrane is partly dependent on electrostatic phage-phage and phage-membrane interactions. If retention was solely due to size exclusion, the greater surface area of the tangential flow membrane should have had a substantial increase in filtration rate without a substantial increase in phage permeability. The key to efficient filtration, at any stage of processing, is the use of a membrane with a large-surface area. However, this can also increase the amount of phage lost to the filtrate, making it important to balance phage retention with filtration speed when choosing the filter size.

The weather and disease data from 2004, 2005, and 2006 paint a fairly complete picture of the disease triangle, as it applies to fire blight in Canada. In general, cool temperatures result in lower disease incidence. However, moderate temperatures do not guarantee infection, since extremely low temperatures immediately following pathogen application can severely limit *E. amylovora* activity, and frost events can cause the death of infected blossoms before *E. amylovora* reaches the base of the peduncle. It is not surprising that the combination of high temperatures and a high inoculum pressure resulted in the complete destruction of a young pear

orchard. The loss of this pear orchard in 2004 prompted a 10-fold reduction in pathogen pressure for the 2005 and 2006 trials. High pathogen pressure may also have prevented the detection of significant treatment effects. Low concentrations of phages were applied in this experiment, but the lack of streptomycin efficacy indicates that an additional factor, common to all treatments, might have masked treatment effects.

Some of the effects of weather can be seen by examining a situation in which a higher pathogen pressure did not lead to a higher incidence of disease. During the 2005 Gala apple trial, about 50% disease developed in the untreated control trees as a result of temperatures that were cool to moderate from the perspective of pathogen growth. By comparison, the daily temperatures in the 2004 Golden Delicious trial were higher, yet virtually no disease developed even though the pathogen pressure was 10-fold higher. These were both apple orchards involving susceptible scion cultivars grafted on to highly susceptible rootstocks. The most notable difference between these two trials was the overnight cold snap that followed pathogen application. This seems to have suppressed the *E. amylovora* population enough to prevent the development of disease during the 2004 season. And yet brown, shepherd's crook-shaped shoot tips were observed the next spring on half of the treated trees. The shepherd's crook is a characteristic sign of necrosis due to prior *E. amylovora* infection, but, unlike true cankers, does not generally harbour living bacteria that can serve as future inoculum. This suggests that these trees were asymptotically infected, probably on the same day that pathogen was applied.

The effect of host susceptibility can be seen by comparing the apple and pear trials in 2005. Data from 2005 suggest that even the mature 12 y old pear trees were inherently more susceptible to infection than the younger apple trees. All 3 orchards were inoculated with *E.*

*amylovora* in the same manner, and experienced identical weather conditions following inoculation. Despite this, a higher incidence of disease was observed among the buffer-treated controls in the pear orchard as compared to the apple orchard. The only difference between the two orchards, other than species, was the bloom stage. However, since blossoms become less susceptible to infection as they age (Hildebrand & Heinicke, 1937; Gouk, Bedford, & Hutshins, 1996; Thomson & Gouk, 2003), this supports the conclusion that the Bartlett pear trees are more susceptible to fire blight than Fulford Gala. In light of this, future experiments in Bartlett pear should be conducted by inoculating treated trees with an even lower concentration of *E. amylovora*, such as a  $1 \times 10^5$  CFU/mL suspension.

The 2005 field trials demonstrate that a phage-mediated biopesticide for fire blight is a practical possibility. Successful phage-carrier treatments reduced the incidence of blossom blight by reducing the population of *E. amylovora* on orchard blossoms to the epiphytic level that was present in the orchard before the start of the experiment. In previous efficacy screens using some of these phages, symptom control on an 11 mm diameter pear fruit plugs was correlated with as much as a 97% reduction in surface *E. amylovora* populations (Gill, 2000). However, control was not always associated with significant population reductions and even those reductions still left as much as  $1 \times 10^6$  to  $1 \times 10^7$  CFU per plug. In the orchard trials described here, successful phage treatment caused a 99% reduction in the *E. amylovora* population, and this reduction was correlated with disease control.

Overall, phage-carrier treatments were no less reliable than the commercially available fire blight biopesticides, and phage-carrier combinations were sometimes effective when the carrier alone or the commercial biopesticides were not. The efficacy of successful treatments was

not statistically distinguishable from the efficacy of streptomycin. Finally, molecular monitoring of the phage, carrier, and pathogen populations allowed the success or failure of selected treatments to be correlated with logical changes in biopesticide and pathogen population sizes, and showed that at least one phage preferentially reproduces at the expense of the pathogen when it is present. In addition, the use of a  $1 \times 10^6$  CFU/mL suspension of *E. amylovora* in 2005 appears to be an appropriate test of fire blight biopesticides, since it produced 50% to 70% disease in the control trees and permitted detection of significant treatment effects.

An average phage population greater than  $1 \times 10^5$  PFU/blossom at the time of pathogen arrival was required to significantly reduce the chance of *E. amylovora* infection. The mean phage population observed in successful phage-based treatments was approximately  $1 \times 10^6$  PFU/blossom, in comparison to  $1 \times 10^5$  PFU/ blossom or less in unsuccessful treatments. Balogh (2002) observed no significant difference in the efficacy of  $1 \times 10^6$  PFU/mL vs.  $1 \times 10^8$  PFU/mL *X. campestris* phages in controlling tomato leaf blight, but these values refer to the initial concentration of phages applied. The actual concentration of active phages on the leaf surfaces was not monitored over the course of the experiments.

*In vitro* replication experiments show that phage-host encounters are a stochastic process dependent on Brownian or externally applied motion. As such, they are dependent upon the presence of a critical density of susceptible host cells in order to replicate, so much so that the traditional calculations of MOI are inappropriate for host concentrations below  $1 \times 10^7$  CFU/mL (Kasman et al., 2002). This may have been a contributing factor in the greater efficacy of phage-carrier treatments that were prepared to  $1 \times 10^8$  rather than  $1 \times 10^6$  CFU or PFU/mL. Initial phage adsorption in the more concentrated preparations would have been consistent with a true MOI of

1, ensuring that enough infected carrier cells reached the blossom surface to initiate a sustainable phage population.

The model presented by Kasman et al (2002), which is based on liquid culture, does not necessarily reflect phage growth patterns once the biopesticide has been applied to the blossom. Interactions among the phages, carrier, and pathogen are likely occurring within the small volumes of aqueous secretions present on the stigma and hypanthium. However, the scale on which this occurs is small enough that growth within an extremely thin agar overlay may be the better analogy: replication within microscopic regions resembles liquid culture, but expansion to adjacent areas is limited the way plaque expansion is limited. It is also possible that neither analogy is appropriate, in which case the population monitoring techniques used in this study may be useful in modeling the population dynamics of the phages and their hosts on plant surfaces.

Several aspects of phage-carrier performance must still be tested in a field situation before this system could be confidently used on a larger scale. All of the phage-carrier biopesticide components were prepared fresh before field use. This strategy is not practical for large-scale production and use. Lyophilization may be an alternative option. Lyophilized bacterial antagonists become established on pear and apple blossom more consistently, and at higher levels, than fresh cultures of the same strains (Stockwell, Johnson, & Loper, 1998). The effect of lyophilizing phages is less certain. *E. amylovora* phages do not seem to survive well when lyophilized in the skim milk formulation, as evidenced by the poor recovery of the Vineland collection (Chapter 2), and the inconsistent recovery of PEa1 and PEa7 from ATCC cultures (A. M. Svircev and J. J. Gill, personal communication). In contrast, many phages tend to

survive well when stored at 4°C in liquid suspension, with little or no loss of viable titre in a year's time (Munsch & Olivier, 1995; Chapter 2).

Since the field trials involving phage cocktails were lost to frost in 2006, these experiments must be repeated at a future date. The phage-carrier biopesticide system must ultimately involve mixtures of several different phage types, which should ideally be tested against a mixed suspension of multiple *E. amylovora* strains. This is partly because cocktails have been shown to reduce the emergence of phage-resistant bacteria (Tanji et al., 2004; Tanji et al., 2005), and partly because phages can be selected based on their ability to broaden the host range of the treatment. However, the success of different phages in experiments involving different host plants at different stages of development suggests that cocktails may improve the reliability of a phage-carrier biopesticide. The rate and efficiency of phage adsorption can be affected by the presence of external cofactors, and by the physiological state of the bacterial host (Guttman, Raya, & Kutter, 2005). Since blossom nectar composition is known to vary with scion cultivar and with blossom age (Paulin, 1987; Pusey, 1999; Pusey & Curry, 2004), it is entirely possible that these factors could influence the efficacy of any one phage by affecting its ability to replicate on the carrier or to attack the pathogen.

### General Summary

The presentation of this research began by emphasizing the importance of integrating multiple performance-based goals into every stage of the biopesticide development process. My application of these principles is to the development of a biopesticide that controls orchard populations of *E. amylovora* through the combined activities of *Erwinia* phages and *P. agglomerans* is summarized here and in Figure D-1.

First, fire blight is an appropriate target for biopesticides in general, and for phage therapy in particular (Chapter 1). The primary point of infection is the open blossom. This is not the only infection court that is important for disease development, but the most common disease stages do follow directly from the blossom blight phase. The open blossom is a surface that is easily accessible using existing pesticide application technology, and its life span represents a finite period of susceptibility. Therefore, phages applied to the blossoms can infect and lyse the pathogen as during the early stages of blossom colonization and growth, thereby preventing both primary infection and the expansion of the total available inoculum in the orchard.

Once an appropriate disease target is selected, the biopesticide must be designed such that it remains active at the necessary site. The blossom surface is, however, a hostile environment for phages. The *P. agglomerans* carrier was used to prolong the period over which infective phages were present by supporting their replication in the absence of the pathogen. New generations of phage were produced at the expense of the carrier, increasing the phage population and limiting the amount of time that free phage were exposed to damaging ultraviolet light and dessication.

The selection of phages and a carrier was based on the simultaneous consideration of factors that would affect all stages of biopesticide production and use (Chapter 2). A *P.*



*agglomerans* carrier was selected that was susceptible to a large number of phages with different host ranges, thereby ensuring that it could eventually support the replication of a mixture of phages that will infect a broad range of *E. amylovora* strains. The carrier also produces an antibiotic against *E. amylovora* which, if active on the blossom, would contribute to pathogen control by antibiosis. Seven phages with broad and differing host ranges were also selected from the Vineland collection. The novelty of the  $\phi$ Ea21-4 genome (Chapter 3) underscores the diversity of both the Vineland phage collection and the global phage metagenome as a whole.

The ability of these phages and carrier candidates to reduce fire blight symptom severity in isolated pear blossoms was also factored into the selection process, since that scenario is representative of the environment in which the biopesticide must be active (Chapter 2). Significant differences in the efficacy of different isolates was rarely obtained in this initial screening process. This was probably the result of treatment timing. Subsequent experiments (Chapter 5) demonstrated that preventative treatment is more likely to be effective when the treatment is applied several hours before inoculation with the pathogen. These later experiments also confirmed that the chosen phages and carrier, when applied in combination, could effectively reduce symptom development in pear blossoms. The significant, though lower, efficacy of the carrier alone showed that it also contributes directly to the control of *E. amylovora* populations on the blossom.

The *in planta* blossom assay was also used to develop the initial parameters for field experiments (Chapter 5). The superior performance of established phage-carrier populations, as well as established protocols for commercial bacterial biopesticides, dictated that treatments should be applied at least one day before the pathogen. The optimum *E. amylovora* inoculum

required for the blossom assays ( $1 \times 10^6$  CFU/mL) was increased ten-fold for the initial field trials based on the disease incidence rates reported for similar pathogen pressures. The destruction of the pear orchard in 2004 showed this to have been an overestimate, and pathogen suspensions were prepared to  $1 \times 10^6$  CFU/mL for subsequent trials (Chapter 7).

To ensure that phage-carrier biopesticide components could be easily produced, the *in vitro* growth characteristics of bacteria and phage were studied, and the results were used to optimize the production of phages for field trials (Chapter 5). Phages that could not be easily grown to high concentrations were removed from consideration. Purification and storage protocols were also developed (Chapter 7).

Finally, the phage-carrier biopesticide was tested in the field (Chapter 7). The results of field trials demonstrate that the tested phage-carrier combinations and application protocols can significantly reduce the incidence of blossom blight in the orchard. Moreover, that reduction is biologically meaningful, having been indistinguishable from the efficacy of streptomycin. These results were achieved by applying a carrier suspension of  $1 \times 10^8$  cfu/mL, with an MOI of 1. This is consistent with previous studies in which phage:bacteria ratios greater than or equal to 10 were required in order to protect plant tissues from disease using pre-treatment with phages (reviewed by Vidaver, 1976). This suggests that a 10:1 ratio of phage:host is a killing ratio, and that a lower ratio would facilitate the long-term persistence of both phage and carrier.

Key to the success of the field trials was the use of multiplex real-time PCR to simultaneously monitor the phage, carrier, and pathogen populations over the course of the experiment. Sampling protocols were developed from *in planta* blossom assays conducted with *E. amylovora* and *E. pyrifoliae* (Chapter 4). With this method it was possible to develop an

understanding of microbial ecology that determines the success or failure of the biopesticide, an understanding which has historically been one of the greatest stumbling blocks in the development of phage-mediated therapies (Goodridge, 2004). The population dynamics elucidated by this method confirmed the hypothesis underlying the design of this biopesticide. Specifically, it was shown that the *P. agglomerans* carrier can support the replication of phages on the blossom surface, and that the phage replicated preferentially on the *E. amylovora* pathogen when it was present. This thesis is the first reported use of an exogenously applied carrier bacterium to directly increase the efficacy of a phage-based therapy. The sequence data obtained from the genome of  $\phi$ Ea21-4 (Chapter 3) will facilitate the development of phage-specific real-time PCR detection so that the relative performance of phages in mixtures can be monitored in future field trials.

The data collected during field trials also demonstrate the complex way in which weather, pathogen pressure, and host susceptibility interact to determine disease outcomes (Chapter 7). One consequence of this variability is that effective fire blight management in Canada may always require that growers have a range of pesticide types available to them: biopesticides for general *E. amylovora* population suppression, and the option of occasional streptomycin applications in those marginal scenarios when the efficacy of biopesticides may be low, but the risk of fire blight is real.

This critical demonstration of efficacy, along with the molecular tools to explain it, are only directly useful for disease control if growers are eventually allowed to use it. Regulatory factors ultimately determine whether a biopesticide is practically useful, regardless of its efficacy. The development of a practical biopesticide therefore requires that the environmental fate of its

components be accounted for, for reasons of human safety and environmental protection.

The safety of the other active component of the phage-carrier biopesticide, the *P. agglomerans* carrier, is not likely to be an issue with respect to either human exposure or potential impacts on orchard ecology. The organism is applied before fruiting, is surface-limited, is naturally present in the orchard at low levels, and has not been shown to be toxic or pathogenic to humans. It is not expected to present any non-occupational exposure risk, and even the occupational risk is minimal. In fact, the US Environmental Protection Agency has granted exemptions to the usual requirement for a maximum permissible residue level of the two commercial biopesticides containing *P. agglomerans*, BlightBan and Bloomtime, stating that “*Pantoea agglomerans* is ubiquitous in the environment, and is recognized as an epiphyte of a wide variety of plants”, and citing extensive evidence that the species is not toxic, pathogenic, or an irritant to animals or humans (Environmental Protection Agency, 2006a; Environmental Protection Agency, 2006b). These products have also been successfully registered in Canada.

Human exposure to phages is not a particular concern. Their specificity for bacterial cells precludes the possibility of human infections, and there is extensive evidence that phages pose no other risk to humans. Phages are the most abundant entities on Earth, and have been found as contaminants in vaccines and sera (Merril et al., 1973; Milch & Fornosi, 1975; Moody, Trousdale, Jorgenson, & Shelokov, 1975). Perhaps most tellingly, the United States Food and Drug Administration recently approved a phage mixture for use on ready-to-eat foods, placing it the “Generally Recognized as Safe” category (EBI Food Safety, 2006).

Still, the fate of exogenously applied *E. amylovora* phages will likely require more study than the fate of *P. agglomerans*. The main concern would be the effect of a high concentration of

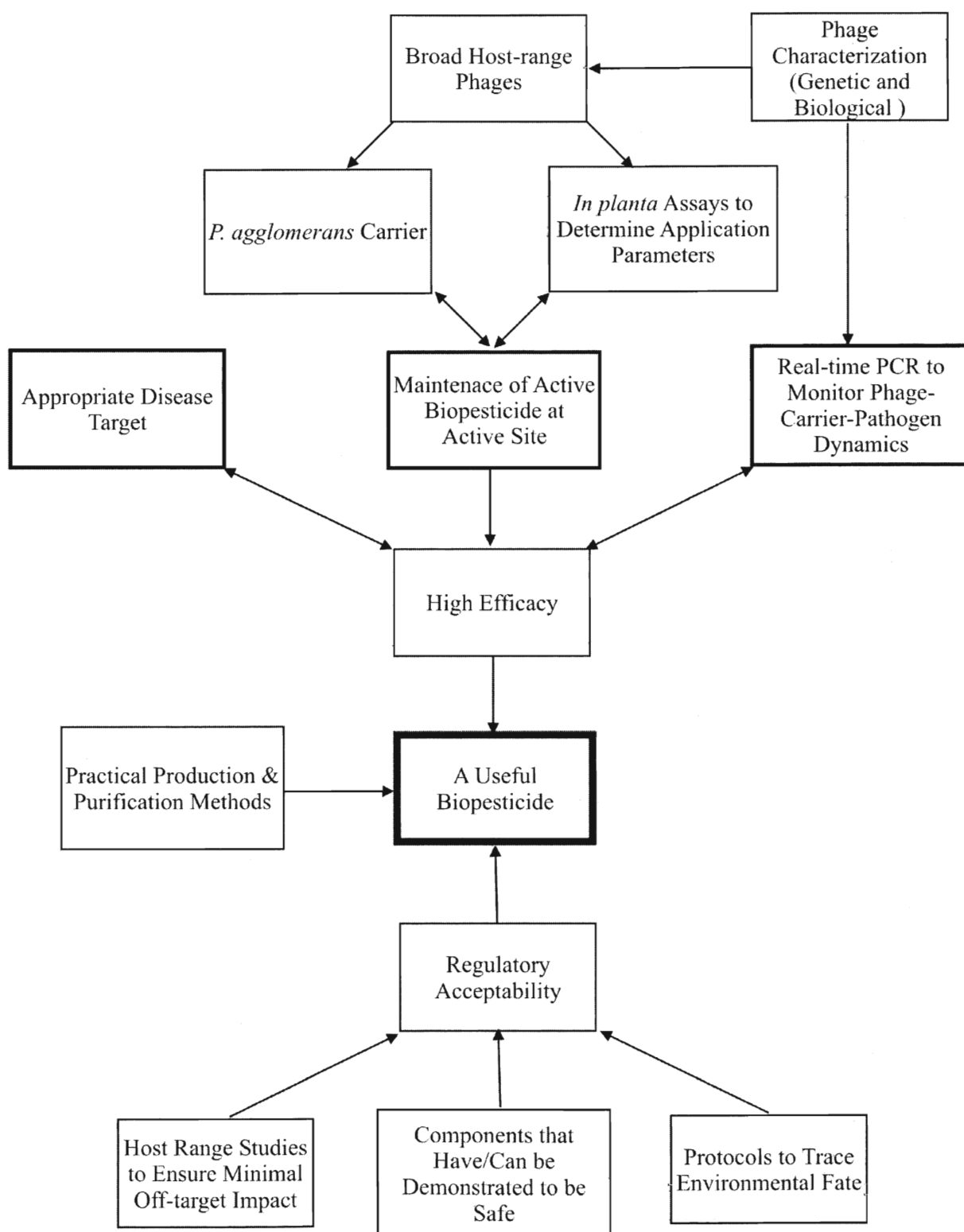


Figure D-1. Integrated development of an effective and practical phage biopesticide.

phages on the overall microbial ecology of the orchard. This was addressed in two ways, by developing a method to track the persistence of phages in soil (Chapter 6), and by confirming that the Vineland phages do not infect other common orchard bacterial genera (Chapter 2).

The research presented in this thesis has implications for the broader study of microbial ecology in soil and in aerial plant tissues and soil. The multiplex real-time PCR protocols that were developed to monitor bacteria and phages in soil samples and on blossoms, in both the field and *in planta* assays, should be applicable to a range of other projects. The ability to monitor natural microbial communities in a completely culture-independent manner is important for our understanding of the role of phages in microbial ecology. Reaney and Marsh (1973) pointed out that if phages occur in soil at even 0.1% of the levels observed in laboratory culture, they must be the most abundant genomes in that environment. Viruses also contribute substantially to biogeochemical cycling (Bratbak, Thingstad, & Haldal, 1994; Middleboe, Jorgensen, & Kroer, 1996), and can facilitate genetic change in their hosts (Holland & Domingo, 1998; Boyd, Davis, & Hochbut, 2001). Enrichment methods not only obscure vital information about population sizes, they restrict studies to phages infecting culturable hosts when only 1% to 5% of endogenous soil bacteria are thought to be culturable at all (Torsvik, Goksøyr, & Daae, 1990).

One response to the unique challenges posed by phage therapy has been to adopt a more reductionist approach and use phage components such as purified or transgenically expressed lethal enzymes (Gaeng et al., 2000; Ko et al., 2002; Salm et al., 2006), lysis-deficient phages which kill bacteria without a sudden increase in endotoxin dispersal (Matsuda et al., 2005), or to simply use inactivated phages as a convenient means of displaying immunogenic epitopes independently of their pathogenic owners (Cao et al., 2000; Solomon, 2007). A unifying theme

among these strategies is that the kinetics of a self-replicating therapeutic agent have been removed from the scenario, returning the treatment to the dose-response relationships associated with classical chemotherapeutic methods. This simplifies many things, but for the most part it does so by sacrificing one of the unique strengths of phages as therapeutic agents. The data presented in this work shows that a phage-based biopesticide is a practical possibility for the control of fire blight, and demonstrates that it is possible to develop a phage-mediated therapy can be developed which capitalizes on, rather than circumvents, the unique properties of phages.

Phage therapy is not suitable for all bacterial diseases, whether in plants or animals. Certain infection courts may not be accessible to phages, as in the case of intracellular mycobacteria, or may not allow them to persist long enough to be effective, as in the bovine udder (Gill et al., 2006a; 2006b). Biofilms also present a particular challenge to phage therapy because of their heterogeneous physical, chemical and biological structure (reviewed by Brüssow & Kutter, 2005). There may be creative solutions to these problems in certain cases, but probably not all of them. At the same time, not all phages are suitable for phage therapy. Phages that carry toxin genes, or that facilitate high rates of transduction, will not be suitable for use in their native form. The success of phage therapy against any particular disease also depends on the availability of many phages strains that, together, are effective against most or all strains of the causative agent. Assuming those factors are favourable, the initial development of the elements of a phage-based biopesticide must be always be conducted with the eventual requirements of the field situation in mind. The population dynamics of the relevant species must also be monitored so that it is possible to develop an understanding of the microbial ecology that determines the success or failure of a treatment in a true *in situ* trial. Finally, the results of those field trials must

be used to identify elements of the biopesticide-pathogen-host plant interactions that can be optimized by further research. As this research has demonstrated, the likelihood of developing practically feasible phage therapies is substantially increased by satisfying these criteria.



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